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(54) Title: MODIFIED CELLS AND METHODS FOR INHIBITING HYPERACUTE REJECTION OF XENOGENEIC TRANSPLANTS

## (57) Abstract

Modified cells for transplantation having reduced capacity to stimulate natural antibody-mediated hyperacute rejection of the cell in a transplant recipient are disclosed. In unmodified form, cells for use in xenogeneic transplantation express at least one epitope on a cell surface antigen which is bound by natural antibodies in a recipient. Prior to transplantation, the cells are modified to alter, reduce or substantially eliminate expression of the epitope on the cell surface. Preferably, the cell is a porcine cell and the epitope is a galactosyl ( $\alpha 1,3$ ) galactose epitope. Methods for reducing the immunogenicity of a cell for transplantation into a recipient are also disclosed. Modified cells of the invention can additionally be treated to alter, reduce or substantially eliminate another surface antigen (e.g., an MHC class I antigen) which stimulates a cellular immune response against the cell in a recipient. Additionally, a recipient can be treated with an agent which inhibits T cell activity, such as an immunosuppressive drug or an anti-T cell antibody.

endothelial cells from aorta  
are treated with enzyme  
L-galactosidase

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## MODIFIED CELLS AND METHODS FOR INHIBITING HYPERACUTE REJECTION OF XENOGENEIC TRANSPLANTS

### Background of the Invention

5           The ability to transplant cells, tissues and organs from animals into humans as replacements for diseased human cells, tissues or organs would overcome a key limitation in clinical transplantation: the shortage of suitable human donor organs. However, the problem of immune-mediated rejection continues to hamper the clinical application of xenogeneic transplantation. Xenogeneic tissues, similar to tissues from mismatched human donors, are  
10       subject to rejection by the human cellular immune system. In addition, when transplanted into a human, cells from nonprimate animals may be rejected by antibodies that are present in human serum even without prior exposure to cells from the animal. The presence of these preexisting, or natural, antibodies results in rapid rejection of xenografts. This mode of destruction of transplanted animal tissues, termed hyperacute rejection, occurs within minutes  
15       to hours of transplantation and is distinct from rejection via the cellular immune pathway which typically occurs over one to several weeks (see Lafferty, K.J. et al. in *Transplantation: Approaches to Graft Rejection*, New York: Alan Liss, 1986, pp. 87-117).

          Natural antibodies in human serum that react with nonprimate animal cells are of both IgM and IgG classes. The binding of natural antibodies to epitopes present on nonprimate  
20       animal cells is thought to mediate hyperacute rejection of the transplanted cells by attraction and activation of complement and/or effector cells that cause cell death. Whole organ grafts can fail even in the absence of antibody-mediated cell death: attack of the endothelial cells lining the vasculature of the xenografted organ can lead to complement-activated release of factors that initiate the coagulation cascade. The anti-thrombogenic lining of the vasculature  
25       is disrupted, resulting in thrombus formation and tissue anoxia, as well as passage of lymphocytes through the endothelial cell layer into the tissue (see Bach, F.H. (1993) *Transpl. Proc.* 25:25-29; and Platt, J.L. and Bach, F. H. (1991) *Transplantation* 52:937-947). The barrier imposed by these naturally occurring antibodies must be surmounted if a transplanted xenogeneic cell, tissue or organ is to engraft successfully.

30           One approach that has been used in an attempt to inhibit hyperacute rejection of a xenograft is to preclear the recipient serum of natural antibodies by perfusion *ex vivo* through one animal organ prior to the transplantation of a second animal organ into the recipient (see Ross, J.R. et al. (1993) *Transplantation* 55:1144-1150; and Tusó, P.J. et al. (1993) *Transplantation* 55:1375-1378). In this procedure, human blood from a recipient is  
35       circulated *ex vivo* through a first pig liver and tested for any residual reactivity with fresh pig tissues prior to transplantation of a second pig liver into the recipient (Tusó, P.J. et al. (1993) *Transplantation* 55:1375-1378). In addition to requiring two donor organs for each transplant recipient, this approach is limited by the efficiency with which natural antibodies can be removed from the circulation of the recipient.

A second approach to inhibiting hyperacute rejection has been to treat the recipient with immunosuppressive drugs or inhibitors of complement prior to transplantation (see Bach, F.H. (1993) *Transpl. Proc.* 25:25-29; and Platt, J.L. and Bach, F.H. (1991) *Transplantation* 52:937-947). This approach has been successful in prolonging the survival of grafts up to several months but suffers from problems generally associated with administration of high doses of immunosuppressants.

Another approach to inhibiting natural antibody-mediated rejection of organ grafts has been to administer high doses of low molecular weight haptens that will inhibit natural antibody binding to the transplanted tissue. This approach has been applied to ABO blood group mismatched allografting, another clinical situation in which pre-existing natural antibodies can mediate hyperacute graft rejection. The survival of an ABO incompatible cardiac allograft was prolonged from minutes to several days by the injection into the graft recipient of synthetic carbohydrate A and B blood group antigens (see Cooper, D.K.C. et al. (1993) *Transplantation* 56:769-777). Studies in humans and nonhuman primates indicate that natural antibodies reactive with cells from a number of different species are directed largely against carbohydrate epitopes on the xenogeneic tissue (see Platt, J.L. (1992) *ASAIO Journal* 8-16; Good, A.H. et al. (1992) *Transpl. Proc.* 24:559-562; and Satake, M. et al. (1993) *Clin. Transpl.* 7:281-288). Thus, Cooper et al. suggested that a similar approach could be used to inhibit hyperacute rejection in xenograft transplantation.

Accordingly, approaches that have been attempted or suggested to address the problem of hyperacute rejection of transplanted cells have been based on treatment of the recipient to remove, suppress or neutralize natural antibodies in the recipient's serum.

#### Summary of the Invention

This invention pertains to inhibition of natural antibody-mediated hyperacute rejection of nonprimate xenografts in human or nonhuman primate transplant recipients to thereby improve xenogeneic engraftment. The methods of the invention are based upon treatment of the graft rather than treatment of the recipient. According to the invention, a cell to be transplanted, which in unmodified form expresses an epitope on its surface which stimulates hyperacute rejection of the cell by natural antibodies in a recipient, is treated such that expression of the epitope on the surface of the cell is altered, reduced or substantially eliminated. This treatment of the graft inhibits subsequent recognition of the epitope by natural antibodies in a recipient, thereby inhibiting hyperacute rejection. In a preferred embodiment, the epitope is a carbohydrate, preferably galactosyl( $\alpha$ 1,3)galactose (Gal( $\alpha$ 1,3)Gal). Preferred cells for treatment include porcine cells. Cell types for use in the invention include endothelial cells, hepatocytes, pancreatic islets, muscle cells (including skeletal and cardiac myocytes and myoblasts), fibroblasts, epithelial cells, neuronal cells, bone marrow cells, hematopoietic cells and lymphoid cells. Dispersed cells can be treated or, alternatively, cells can be treated within a tissue or organ (e.g., liver, heart, kidney etc.).

In one embodiment of the invention, natural antibody epitopes are removed from the surface of a cell, such as by enzymatic or chemical treatment of the cell. For example, Gal( $\alpha$ 1,3)Gal epitopes can be cleaved from a cell surface by treatment of the cell with an alpha-galactosidase. In another embodiment, formation of the epitope on the cell surface is inhibited. This can be accomplished by inhibiting the activity of an enzyme which forms the epitope. For example, formation of Gal( $\alpha$ 1,3)Gal epitopes on the surface of a cell can be interfered with by inhibiting the activity of an alpha-1,3-galactosyltransferase within the cell, such as by introducing into the cell a nucleic acid which is antisense to a coding or regulatory region of an alpha-1,3-galactosyltransferase gene or by treating the cell with a chemical inhibitor of the enzyme. In yet another embodiment, epitopes on a cell surface are altered by binding a molecule to the epitope, thereby inhibiting its subsequent recognition by natural antibodies in a recipient. For example, lectins, antibodies or antibody fragments can be bound to an epitope to inhibit its subsequent recognition by natural antibodies.

Accordingly, one aspect of the invention pertains to a cell for transplantation into a recipient, which, in unmodified form expresses at least one epitope on a cell surface antigen that is bound by natural antibodies in a recipient, wherein the cell is modified to alter, reduce or substantially eliminate expression of the epitope on the cell surface. In a preferred embodiment, the cell is a porcine cell and the epitope is a Gal( $\alpha$ 1,3)Gal epitope.

In addition to being modified to alter, reduce or substantially eliminate expression of at least one natural antibody epitope, the cell can be further modified to alter, reduce or substantially eliminate expression of at least one other antigen on the cell surface which, in unmodified form, stimulates a cellular immune response against the cell in a recipient. A preferred second antigen to be altered, reduced or substantially eliminated on the cell is an MHC class I antigen. For example, the cell can be further modified by contacting the cell, prior to transplantation, with an anti-MHC class I antibody, or fragment thereof (e.g., F(ab')<sub>2</sub> fragment). In another embodiment, the cell is further modified to express a gene product, for example by introducing into the cell a nucleic acid encoding the gene product (e.g., for gene therapy).

Another aspect of the invention pertains to methods for reducing the immunogenicity of a cell for transplantation. The methods involve contacting the cell with a first agent which alters, reduces or substantially eliminates expression of a natural antibody epitope on the cell surface. The agent can be, for example, an enzyme which cleaves the epitope from the cell surface, an antisense nucleic acid which inhibits formation of the epitope on the cell surface or a molecule which binds to the epitope on the cell surface and inhibits its recognition by natural antibodies in a recipient. In addition to the first agent, the cell can be contacted with another agent to alter, reduce or substantially eliminate another antigen on the cell surface which, in unmodified form, stimulates a cellular immune response against the cell (e.g. an MHC class I antigen). Following treatment of the cell to reduce its immunogenicity, the cell is administered to a recipient. In addition to receiving a cell having reduced immunogenicity,

a transplant recipient can also be treated with another agent which inhibits T cell activity in the recipient (e.g., an immunosuppressive drug or anti-T cell antibody) to further inhibit rejection of the transplanted cells.

5 **Brief Descriptions of the Drawings**

*Figures 1A-1D* are graphic representations of the reactivity of human and monkey sera with untreated porcine endothelial cells.

*Figure 2* is a graphic representation of the effect of alpha-galactosidase treatment on the viability of porcine endothelial cells.

10 *Figures 3A-3F* are graphic representations of the binding of natural antibodies to porcine endothelial cells after alpha galactosidase treatment of the cells.

*Figure 4* is a graphic representation of the time course for the reappearance of alpha galactosyl epitopes on porcine endothelial cells following treatment of the cells with alpha-galactosidase.

15 *Figure 5* is a graphic representation of the binding of various human and animal sera to porcine endothelial cells, either untreated or treated with alpha galactosidase to remove alpha galactosyl epitopes, demonstrating reduced binding of human and monkey IgG and IgM to porcine cells after enzyme treatment.

20 *Figure 6* is a graphic representation of the effect of various concentrations of human serum and complement on the viability of porcine endothelial cells.

*Figure 7* is a graphic representation of the variation in levels of natural antibodies in human serum from seven individuals.

25 *Figure 8* is a graphic representation of the viability of porcine endothelial cells upon incubation with bovine or human sera either in the presence or absence of complement, demonstrating natural antibody-mediated cytotoxicity of human serum and complement.

*Figure 9* is a graphic representation of the viability of porcine endothelial cells, either untreated or treated with alpha galactosidase, upon incubation with human serum and complement, demonstrating increased viability of the porcine cells following enzyme treatment.

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**Detailed Description of the Invention**

This invention features methods for inhibiting hyperacute rejection of transplanted cells by preformed natural antibodies in a transplant recipient. Modified cells for use in transplantation are provided which have a reduced capacity to stimulate hyperacute rejection in a recipient. Cells are modified such that expression of at least one epitope on the surface of the cell which stimulates hyperacute rejection (i.e., at least one natural antibody epitope) is altered, reduced or substantially eliminated. The modified cells of the invention are particularly useful in xenogeneic transplantation, where natural antibody-mediated hyperacute rejection has posed a barrier to successful transplantation. Modified cells of

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nonprimate organ can thus be transplanted into humans and nonhuman primates. The modified cells and methods of the invention are also applicable in certain allogeneic transplant situations in which natural antibody-mediated hyperacute rejection may occur, such as with ABO blood group mismatches.

5       The term "hyperacute rejection", as used herein, refers to an immunological reaction in a recipient against foreign cells which occurs very rapidly (e.g., within minutes to hours) following transplantation of the cells. Typically, hyperacute rejection is mediated by natural antibodies present in the serum of the recipient.

10       An individual who has received or is to receive a foreign cell, tissue or organ graft is referred to herein as a "recipient" (or "host"). An individual that supplies the foreign cell tissue or organ graft is referred to herein as a "donor".

15       The term "natural antibody" as used herein refers to preformed (i.e., preexisting) antibodies reactive against foreign donor cells which are present in serum of a recipient without prior exposure to donor cell antigens. A structure on the surface of a donor cell (e.g., a carbohydrate) which is specifically recognized by (i.e., can be bound by) a natural antibody is referred to herein as a "natural antibody epitope" or simply an "epitope".

20       To produce modified cells within the scope of the invention, the expression of at least one epitope on the surface of the cell to be transplanted which stimulates hyperacute rejection of the cell by natural antibodies in a recipient is altered, reduced or substantially eliminated. The terms "altered" or "alteration" of the expression of an epitope is intended to encompass modification of the epitope such that its recognition by natural antibodies is inhibited or prevented (although the epitope may still be present at normal levels on the cell surface). Alternatively, when expression of the epitope is "reduced" or "substantially eliminated", the level of expression of the epitope on the cell surface (i.e., amount of the epitope on the cell surface) is decreased or substantially abolished relative to a normal level of expression of the epitope on the cell surface.

25       Various aspects of the invention are described in further detail in the following subsections:

30       I. Epitopes to be Altered, Reduced or Substantially Eliminated

35       Epitopes to be altered, reduced or substantially eliminated according to the invention are those which stimulate hyperacute rejection of a cell in a recipient. Since natural antibodies in humans and nonhuman primates are predominantly directed against carbohydrate epitopes, preferred epitopes for modification are carbohydrate moieties. A preferred carbohydrate epitope to be altered, reduced or substantially eliminated is a galactosyl( $\alpha$ 1,3) galactose epitope (also referred to herein as Gal( $\alpha$ 1,3)Gal or an alpha-galactosyl epitope). Up to 1 % of all circulating IgG in human sera has been found to be directed against this epitope (see Galili, U. et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:1369-1373; Galili, U. et al. (1987) *J. Biol. Chem.* 262:4683-4688). An experiment described in

Example 2 demonstrates that this epitope on porcine cells is a major epitope recognized by natural antibodies in human serum. Terminal alpha-galactosyl epitopes are present on the surface of cells from nonprimate mammals, prosimians and New World monkeys but not on the surface of cells from humans, apes and Old World monkeys (see Galili, U. et al. (1988) *Proc. Natl. Acad. Sci. USA* 263:17755-17762). The term "galactosyl( $\alpha$ 1,3) galactose epitope" is intended to encompass carbohydrate structures which comprise this moiety and which can be bound by natural antibodies, such as  $\alpha$ Gal(1-3) $\beta$ Gal(1-4) $\beta$ GlcNAc and  $\alpha$ Gal(1-3) $\beta$ Gal(1-4) $\beta$ Glc (collectively referred to as linear B type structures) (for descriptions of natural antibody binding to various alpha-galactosyl epitopes see Good, A.H. et al. (1992) *Transpl. Proc.* 24:559-562).

Alternatively, other epitopes recognized by natural antibodies can be targeted for alteration, reduction or elimination. Other carbohydrate epitopes that have been reported to be bound by natural antibodies include A or A-like carbohydrates (including A disaccharide, A trisaccharide, A type 4, A type 5, A type 6 and linear A type 6), Forssman disaccharide and Forssman trisaccharide,  $\alpha$ -L-Rhamnose and N-acetyl  $\beta$ -D-glucosaminide, as described in Good, A.H. et al. (1992) *Transpl. Proc.* 24:559-562; and sulphatides such as galactosylceramide-3-sulphate and lactocylceramide-3-sulphate, as described in Holgersson, J. et al. (1992) *Transpl. Proc.* 24:605-608. Additionally, glycoproteins having molecular weights of 115 kD, 125 kD and 135 kD have been reported to express epitopes recognized by natural antibodies (see Platt, J.L. et al. (1990) *Transplantation* 50:817-822).

## II. Methods for Altering, Reducing or Eliminating a Cell Surface Epitope

According to the invention, a cell for transplantation is treated prior to transplantation to alter, reduce or substantially eliminate expression of at least one epitope on the cell surface that stimulates hyperacute rejection of the cell in a recipient. In a preferred embodiment of the invention, expression of a surface epitope is reduced or substantially eliminated by removing the epitope from the cell surface. Removal of the natural antibody epitope from the cell surface inhibits subsequent recognition of the cell by natural antibodies in a transplant recipient. An epitope can be removed from the surface of a cell by treating the cell with an enzyme or chemical which cleaves the epitope from the surface of the cell. For example, carbohydrate epitopes can be cleaved from a cell surface by treatment of the cell with one or more endo- or exoglycosidases specific for the carbohydrate to be cleaved. Preferably, alpha-galactosyl epitopes are cleaved from a cell surface by treatment of the cell with an alpha-galactosidase. As described in greater detail in Example 1, treatment of cells *in vitro* prior to transplantation with an alpha-galactosidase (e.g., coffee bean alpha-galactosidase; commercially available from Sigma Chemical Co., St. Louis, MO) removes surface alpha-galactosyl epitopes. Following treatment, removal of these epitopes from the cell surface can be assessed, for example, by reacting the cells, with a labelled lectin specific for the alpha-galactosyl epitope (e.g., *Griffonia simplicifolia*, or GS-1, lectin; commercially available from



EY Labs) and assessing the amount of binding of the labelled lectin to the treated cells compared to untreated control cells (see Example 1). As described in the Examples, it has been found that GS-1 binding activity on the surface of porcine endothelial cells is undetectable after alpha-galactosidase treatment. Moreover, it has been found that alpha-galactosyl epitopes on the cell surface are not reexpressed for several hours after treatment and that even 48 hours after treatment, GS-1 binding activity is still diminished by 60%. Thus, alpha-galactosidase treatment of cells is sufficient to remove surface alpha-galactosyl epitopes and this treatment leads to prolonged diminution of expression of these epitopes on the cell surface. Furthermore, alpha-galactosidase treatment greatly (i.e., >90%) inhibits natural antibody-mediated (human or monkey), complement dependent lysis of the porcine endothelial cells (see Example 3). In addition to alpha-galactosidase treatment, other carbohydrate moieties can be cleaved by a glycosidase having specificity for that moiety. Alternatively, a chemical treatment which removes one or more specific carbohydrate moieties, while retaining cell viability and function, can be used to remove natural antibody epitopes from the surface of a cell.

To remove cell-surface natural antibody epitopes, a cell is treated with an amount of enzyme (or chemical) and for a period of time sufficient to reduce or substantially eliminate expression of the epitope on the cell surface such that upon transplantation of the cell into a recipient hyperacute rejection of the cell is inhibited. Appropriate dosages and digestion times may vary depending, for example, upon the cell type being treated and the type of digestion reagent used. Appropriate digestion conditions can easily be determined by one skilled in the art according to the teachings of the invention. A non-limiting example of digestion conditions for removal of surface alpha-galactosyl epitopes is 500 milliunits of coffee bean alpha-galactosidase (Sigma Chemical Co., St. Louis, MO) per  $1 \times 10^6$  cells for 2 hours at 37 °C in a buffer of 200 mM sodium acetate in phosphate buffered saline (PBS) (pH 5.8).

In another embodiment of the invention, expression of a cell surface natural antibody epitope is reduced or substantially eliminated by inhibiting or preventing formation of the epitope on the cell surface, e.g., by interfering with the synthesis of the epitope. For example, the activity of an enzyme within the cell which is necessary for formation of the epitope can be inhibited. Carbohydrate moieties are typically attached to glycoproteins or glycolipids by specific glycosyltransferases. Thus, expression of a carbohydrate epitope on a cell surface can be reduced or substantially eliminated by inhibiting the activity of a glycosyltransferase involved in the synthesis of the epitope. For example, the enzyme responsible for attaching galactose in alpha linkage to an underlying chain of sugars on both glycoproteins and glycolipids is UDP galactose alpha-1,3-galactosyltransferase (also referred to herein as alpha-galactosyltransferase). The enzyme substrate specificity and kinetics of this enzyme have been studied (see Elices, M.J. and Goldstein, I.J. (1989) *J. Biol. Chem.* 264:1375-1380; and Joziassse, D.H. et al. (1987) *J. Biol. Chem.* 262:2025-2033) and the gene for the enzyme has

been cloned from bovine (Joziassse, D.H. et al. (1989) *J. Biol. Chem.* 264:14290-14297) and murine (Larsen, R.D. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:8227-8231) tissues. A gene is present in humans that displays considerable homology to the murine gene, but, due to a frameshift mutation and several nonsense mutations in the human counterpart of the murine gene (Larsen, R.D. et al. (1990) *J. Biol. Chem.* 265:7055-7061), the active enzyme is not synthesized in humans and Old World monkeys. As a result, this carbohydrate epitope which is recognized by the natural antibodies in human serum is not normally present on human cells.

In a preferred embodiment, the activity of a glycosyltransferase, e.g. an alpha-galactosyltransferase, is inhibited by introducing into a cell a nucleic acid which is antisense to a regulatory or coding region of the glycosyltransferase gene, thereby repressing transcription of the gene or translation of the mRNA. An "antisense" nucleic acid molecule comprises a nucleotide sequence which is complementary to a coding strand (i.e., sense strand) of another nucleic acid, e.g. complementary to an mRNA sequence, constructed according to the rules of Watson and Crick base pairing, and thus can hydrogen bond to the sense strand of the other nucleic acid. An antisense nucleic acid can form a duplex with an mRNA strand and prevent its efficient translation. Additionally, antisense nucleic acids may increase RNase-mediated degradation of mRNA and/or inhibit splicing of pre-mRNA. An antisense sequence can be complementary to a sequence found in the coding region of an mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. To inhibit translation, the antisense nucleic acid is preferably complementary to a region preceding or spanning the translation initiation codon. Alternatively, an antisense nucleic acid can bind to DNA to form a triple helix and prevent gene transcription (see e.g., Stein, C.A. and Cheng Y.-C. (1993) *Science* 261:1004-1012). Thus, an antisense nucleic acid can be complementary in sequence to a regulatory region of a gene encoding a glycosyltransferase, for instance complementary to a transcription initiation sequence or regulatory element (e.g., promoter or enhancer sequence). For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., *Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics*, Vol. 1(1) 1986.

In one embodiment, a nucleic acid which is antisense to a regulatory or coding region of a glycosyltransferase gene (e.g., alpha-galactosyltransferase) is an oligonucleotide. Typically, oligonucleotides between about 5 and 50 nucleotides in length are used. More preferably, oligonucleotides between about 5 and 35 nucleotides are used. Even more preferably, oligonucleotides about 20 nucleotides in length are used. An antisense oligonucleotide can be constructed using chemical synthesis procedures known in the art. An oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the oligonucleotide or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids. For example, phosphorothioate, methyl phosphonate and

ethyl phosphotriester antisense oligonucleotides (reviewed in Stein, C.A. and Cheng Y-C. (1993) *Science* 261:1004-1012) are within the scope of the invention. Additionally, acridine substituted nucleotides can be used in the antisense oligonucleotides of the invention.

Antisense oligonucleotides can be used to inhibit the activity of a glycosyltransferase in a cell by incubating them with the cell *in vitro* and/or administering them to a subject at an amount and for a time period sufficient to inhibit transcription of the glycosyltransferase gene or translation of the glycosyltransferase mRNA in the cell (see Example 4).

In another embodiment, an antisense nucleic acid is produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., nucleic acid transcribed from the inserted sequence will be in an antisense orientation relative to a target nucleic acid of interest). The antisense expression vector is introduced into cells, for example, in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region of the vector, the activity of which can be determined by the cell type into which the vector is introduced. Preferably, the recombinant expression vector is a recombinant viral vector, such as a retroviral, adenoviral or adeno-associated viral vector. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include  $\psi$ Crip,  $\psi$ Cre,  $\psi$ 2 and  $\psi$ Am. Adenoviral vectors are described in Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Adeno-associated vectors (AAV) are reviewed in Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129. An example of a suitable AAV vector is described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260.

A recombinant expression vector containing a nucleic acid in an antisense orientation is introduced into a cell to generate antisense nucleic acids in the cell to thereby inhibit the activity of a glycosyltransferase in the cell. The vector can be introduced into a cell by a conventional method for introducing nucleic acid into a cell. When a viral vector is used, the cell can be infected with the vector by standard techniques. Cells can be infected *in vitro* or *in vivo*. When a non-viral vector, e.g., a plasmid, is used, the vector can be introduced into the cell by, for example, calcium phosphate precipitation, DEAE-dextran transfection, electroporation or other suitable method for transfection of the cell.

In yet another embodiment, an antisense nucleic acid used to inhibit a glycosyltransferase activity in a cell is a ribozyme which is capable of cleaving a single-stranded nucleic acid encoding the glycosyltransferase, such as an mRNA transcript. A

catalytic RNA (ribozyme) having ribonuclease activity can be designed which has specificity for an mRNA encoding a glycosyltransferase, such as an alpha-galactosyltransferase. For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in an alpha-galactosyltransferase mRNA. See for example Cech et al. U.S. Patent No. 4,987,071; Cech et al. U.S. Patent No. 5,116,742 for descriptions of designing ribozymes. Alternatively, an alpha-galactosyltransferase RNA can be used to select a catalytic RNA having specific ribonuclease activity against the alpha-galactosyltransferase RNA from a pool of RNA molecules. See for example, Bartel, D. and Szostak, J.W. *Science* **261**:1411-1418 (1993) for a description of selecting ribozymes. A ribozyme can be introduced into a cell by constructing a recombinant expression vector (e.g., a viral vector as discussed above) containing nucleic acid which, when transcribed, produces the ribozyme (i.e., DNA encoding the ribozyme is cloned into a recombinant expression vector by conventional techniques).

A preferred antisense nucleic acid of the invention is antisense to a coding or regulatory region of an alpha-galactosyltransferase gene. Antisense oligonucleotides, or an antisense recombinant expression vector can be designed based upon nucleotide sequences of alpha-galactosyltransferase cDNAs known in the art. The nucleotide sequence of a murine  $\alpha$ -1,3-galactosyltransferase cDNA is disclosed in Larsen, R.D. (1989) *Proc. Natl. Acad. Sci. USA* **86**:8227-8231. The nucleotide sequence of a bovine  $\alpha$ -1,3-galactosyltransferase cDNA is disclosed in Joziase, D.H. et al. (1989) *J. Biol. Chem.* **264**:14290-14297. To inhibit the activity of an alpha-galactosyltransferase in a cell from a species other than mouse or cow (e.g., pig), antisense oligonucleotides are designed which are complimentary to nucleotide sequences that are conserved among alpha-galactosyltransferase genes in different species (e.g., based upon comparison of the murine and bovine sequences to identify conserved regions). Additionally, the known murine and bovine cDNA sequences can be used to design hybridization probes or PCR primers which allow isolation of cDNA and/or genomic DNA clones of alpha-galactosyltransferases from other species (e.g., pig) by standard techniques. An antisense nucleic acid for use in the invention can then be designed based upon the nucleotide sequence of a cDNA or genomic DNA fragment so isolated. Suitable antisense oligonucleotides for inhibiting the activity of an alpha-galactosyltransferase in a cell, designed based upon the murine and bovine alpha-galactosyltransferase cDNA sequences, are described in Example 4 and shown in SEQ ID NOS:1-6.

Alternative to antisense nucleic acids, the activity of a glycosyltransferase in a cell can be inhibited, for example, by use of a competitive mono-, di- or oligosaccharide inhibitor or other form of chemical inhibitor of the enzyme. A mono-, di- or oligosaccharide which mimics the carbohydrate moiety that is transferred by the transferase enzyme but which cannot be attached to glycoproteins or glycolipids can be added in excess to cells to competitively inhibit the activity of the glycosyltransferase in the cells. For example, an alpha-1,3-galactosyl transferase in cells can be inhibited by incubating the cells in culture

with soluble  $\alpha$ -methyl-D-galactoside or  $\alpha$ -Gal(1,3) $\beta$ -Gal(1,4)Glc. Alternatively, a non-carbohydrate chemical inhibitor of a glycosyltransferase can be used. An *in vitro* assay can be used to screen for chemical inhibitors of a specific glycosyltransferase. For example, an inhibitor of an alpha-galactosyltransferase can be identified based upon the ability of the compound to inhibit the transfer of galactose to an N-acetyllactosamine acceptor using a procedure such as that described in Cummings, R.D. and Mattox, S.A. (1988) *J. Biol. Chem.* 263:511-519. To inhibit the activity of an alpha-galactosyltransferase in a cell capable of expressing alpha-galactosyl epitopes, a chemical inhibitor so identified can then be contacted with the cell.

10 An alternative approach to inhibiting the activity of an alpha-galactosyltransferase in a cell is to mutate or substantially eliminate the gene encoding the enzyme in the cell, such as by homologous recombination, thereby preventing expression of an alpha-galactosyltransferase in the cell. For example, a homologous recombinant animal can be created in which the alpha-galactosyltransferase gene has been mutated or disrupted. The term "homologous recombinant animal" as used herein is intended to describe an animal containing a gene which has been modified by homologous recombination between the gene and a DNA molecule introduced into an embryonic cell of the animal. To create a homologous recombinant animal, a vector is prepared which contains a portion of the alpha-galactosyltransferase gene which has been mutated or disrupted, flanked at its 5' and 3' ends by additional regions of the alpha-galactosyltransferase gene of sufficient length for successful homologous recombination to occur between the mutated gene contained within the vector and an endogenous wild-type alpha-galactosyltransferase gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous gene are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harbouring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA. Such a homologous recombinant animal, in which an alpha-galactosyltransferase gene has been mutated or disrupted, can be used as a source of donor cells for transplantation that have a reduced capacity to stimulate hyperacute rejection of the cells in a recipient.

In yet another embodiment of the invention, expression of a cell surface natural antibody epitope is altered by binding a molecule to the epitope such that subsequent binding

of natural antibodies to the epitope in a recipient is inhibited (i.e., recognition of the epitope by natural antibodies in a recipient is blocked or obscured by the binding of another molecule to the epitope). For example, a lectin which binds to a specific carbohydrate epitope can be used to alter that epitope on the surface of a cell prior to transplantation. A preferred lectin for altering alpha-galactosyl epitopes is the *Griffonia simplicifolia* I lectin (referred to herein as GS-1) which specifically binds to this carbohydrate structure. GS-1 lectin is commercially available from Vector Laboratories, Burlingame, CA. Other lectins which specifically recognize carbohydrate moieties are known in the art. A cell to be transplanted which expresses alpha-galactosyl epitopes can be incubated prior to transplantation with GS-1 lectin in an amount and for a period of time sufficient to alter cell surface alpha-galactosyl epitopes such that, upon transplantation, binding of natural antibodies in the recipient to the epitopes is inhibited.

Alternatively, an antibody, or fragment thereof, which binds to the epitope but does not activate complement or induce lysis of the cell in a recipient can be used to alter the epitope on the cell surface. Such an antibody, or fragment thereof, competitively inhibits the binding of natural antibodies to the epitope. Polyclonal antibodies or, more preferably, monoclonal antibodies can be used. A mouse monoclonal antibody, termed Gal-13, which specifically recognizes alpha-galactosyl epitopes has been described in the art (see Galili, U. et al. (1987) *J. Biol. Chem.* 262:4683-4688). Monoclonal antibodies specific for alpha-galactosyl epitopes or other carbohydrate epitopes can be prepared by standard techniques known in the art. Preferably, an  $F(ab')_2$  fragment specific for the epitope is used to alter the epitope, thereby avoiding activation of complement in a recipient.  $F(ab')_2$  fragments can be prepared from intact antibodies by conventional techniques, such as pepsin treatment. In a standard procedure for generating  $F(ab')_2$  fragments, intact antibodies are incubated with immobilized pepsin and the digested antibody mixture is applied to an immobilized protein A column. The free Fc portion binds to the column while the  $F(ab')_2$  fragments pass through the column. The  $F(ab')_2$  fragments can be further purified by HPLC or FPLC.  $F(ab')_2$  fragments can be treated to reduce disulfide bridges to produce Fab' fragments. A cell to be transplanted can be incubated prior to transplantation with an antibody, or fragment thereof, which binds to a cell surface natural antibody epitope in an amount and for a period of time sufficient to alter the surface epitope such that, upon transplantation, binding of natural antibodies to the epitope in the recipient is inhibited.

Treatment of a cell to be transplanted (or treatment of cells within a tissue or organ) according to one of the above-described approaches results in altered, reduced or substantially eliminated expression of at least one cell surface natural antibody epitope. The degree of reduction of expression of the epitope on the cell surface (e.g., by treatment with a glycosidase or a nucleic acid which is antisense to a glycosyltransferase gene) can be determined by assessing the amount of the epitope expressed on the cell surface following treatment. This can be accomplished, for example, by incubating the cell after treatment with

a labelled lectin or antibody which specifically recognizes the epitope on the cell surface and assessing the amount of labelled lectin or antibody bound to the cell compared to untreated control cells. A fluoroisothiocyanate-labelled GS-1 lectin (commercially available from Vector Laboratories, Burlingame, CA) can be used to assess the level of expression of alpha-galactosyl epitopes on the surface of treated cells prior to transplantation, such as described in Example 1. Preferably, the level of expression of the epitope is reduced by at least 50-70 %, more preferably at least 70-90%, and even more preferably greater than 90% compared to untreated cells. In the most preferred embodiment, the expression of the epitope on the cell surface is substantially eliminated such that the presence of the epitope on the cell surface is no longer detectable by standard techniques (at least temporarily, although the treatment may not result in permanent removal of the epitope from the cell surface).

It may not be necessary to permanently alter, reduce or substantially eliminate the expression of natural antibody epitopes on the surface of transplanted cells in order to inhibit rejection of the cells in a host due to a phenomenon that has been termed accommodation (for a discussion see Platt, J.L. et al. (1990) *Immunology Today* 11:450-456). It has been demonstrated that temporary depletion of natural antibodies from the circulation of a transplant recipient can be sufficient to enable prolonged survival of the graft, despite the eventual reappearance of natural antibodies in the circulation (see Alexandre, G.P.J. et al. in *Xenograft* 25, Hardy, M.A. (ed.) New York:Elsevier Science Publishers, 1989, pp. 259-266; and Platt, J.L. et al. (1991) *Transplantation* 52:214-220). Thus, temporary alteration, reduction or elimination of expression of the epitope on the surface of cells for transplantation may be sufficient to escape the initial hyperacute response against the graft and killing of the transplanted cells by a natural antibody-mediated mechanism. In the absence of this initial rejection episode, accommodation may occur such that expression of new epitopes on the cell surface, in the presence of natural antibodies in the host, will not induce rejection.

### III. Cells for Transplantation

One aspect of the invention relates to a modified cell suitable for transplantation. Cells which can be modified in accordance with the methods include those which express at least one epitope on a cell surface which stimulates hyperacute rejection of the cell by natural antibodies when the cell is transplanted into a recipient. According to the invention, the cell is modified to alter, reduce or substantially eliminate expression of the epitope on the surface of the cell, thereby inhibiting hyperacute rejection of the cell when transplanted into a recipient. Expression of the epitope on the surface of the cell is altered, reduced or substantially eliminated by one or more of the above-described treatments. Accordingly, in various embodiments, the epitope is removed from the cell surface, the formation of the epitope on the cell surface is inhibited or the epitope is altered by contacting the cell prior to transplantation with at least one molecule which binds to the epitope.

Cells for use in this invention encompass cell types which can be transplanted for therapeutic purposes and which are capable of expressing on their surface at least one epitope which is recognized by natural antibodies in a recipient. Examples of such cells include endothelial cells, hepatocytes, pancreatic islet cells, muscle cells (including skeletal and cardiac myocytes and myoblasts), fibroblasts, epithelial cells, neuronal cells, bone marrow cells, hematopoietic cells and lymphoid cells from nonprimate mammals and certain primates (e.g., prosimians and New World Monkeys).

In a preferred embodiment, the epitope on the cell which is altered, reduced or substantially eliminated is a galactosyl( $\alpha$ 1-3)galactose epitope. Cells which are capable of expressing alpha-galactosyl epitopes include cells from nonprimate mammals (e.g., pigs), prosimians and New World Monkeys (see Galili, U. et al. (1988) *J. Biol. Chem.* 263:17755-17762). Preferably, the cell is a porcine cell. In one embodiment, expression of alpha-galactosyl epitopes on a cell surface is reduced or substantially eliminated by introducing into the cell a nucleic acid which is antisense to a regulatory or coding region of an alpha-galactosyl-transferase gene (e.g., a pig alpha-galactosyltransferase gene in a porcine cell), as described above. Accordingly, the invention encompasses a cell which has been modified to contain such an antisense nucleic acid, e.g., an oligonucleotide or a recombinant expression vector (for example, a retroviral, adenoviral or adeno-associated vector).

Natural antibody epitopes can be altered, reduced or substantially eliminated on the surface of a dispersed population of cells which are to be transplanted into a recipient. Alternatively, a tissue or organ to be transplanted can be treated to alter, reduce or substantially eliminate the expression of natural antibody epitopes on the surface of cells within the tissue or organ. For example, alpha-galactosyl epitopes can be removed from the surface of cells within a tissue or organ by incubating the tissue in a solution containing an alpha-galactosidase or by perfusing the organ with a solution containing the alpha-galactosidase. Alternatively, a tissue or organ can be contacted with (e.g., incubated with or perfused with) an oligonucleotide antisense to a glycosyltransferase gene, or infected with a viral vector containing nucleic acid antisense to a glycosyltransferase gene, to inhibit the activity of an alpha-galactosyltransferase in the cells within the tissue or organ. Accordingly, the invention is not only applicable to transplantation of dispersed cells, but also to transplantation of intact tissues and whole organs, such as heart, liver, kidney, lung, pancreas, stomach, intestines, skin and muscle tissue.

Further modifications of a cell of the invention, in addition to alteration, reduction or elimination of at least one cell surface natural antibody epitope, are within the scope of the invention. For example, in addition to modifying a cell of the invention to inhibit hyperacute rejection of the cell in a recipient, the cell can also be modified to inhibit a cellular immune response against the cell in a recipient. Accordingly, at least one antigen on the cell surface which stimulates a cellular immune response against the cell in a recipient can be altered prior to transplantation. A preferred antigen on the cell surface to be altered is an MHC class



I antigen. In a one embodiment, the antigen on the donor cell to be altered is an MHC class I antigen.

- At least two different epitopes on the same MHC class I antigen on the donor cell can be altered prior to transplantation. MHC class I antigens are present on almost all cell types.
- 5 In a normal immune response, self MHC molecules function to present antigenic peptides to the T cell receptor (TCR) on the surface of self T lymphocytes. In immune recognition of allogeneic or xenogeneic cells, foreign MHC antigens (most likely together with a peptide bound thereto) on donor cells are recognized by the T cell receptor on host T cells to elicit an immune response. Epitopes on an MHC class I antigen on a donor cell are altered to interfere
- 10 with recognition of the MHC class I antigen by T cells in an allogeneic or xenogeneic host (e.g., portions of the MHC class I antigen which are normally recognized by the T cell receptor are blocked or "masked" such that normal recognition of the MHC class I antigen can no longer occur). Additionally, an altered form of an MHC class I antigen which is exposed to host T cells (i.e., available for presentation to the host T cell receptor) may deliver
- 15 an inappropriate or insufficient signal to the host T cell such that, rather than stimulating an immune response against the allogeneic or xenogeneic cell, donor cell-specific T cell non-responsiveness is induced. For example, it is known that T cells which receive an inappropriate or insufficient signal through their T cell receptor (e.g., by binding to an MHC antigen in the absence of a costimulatory signal, such as that provided by B7) become anergic
- 20 rather than activated and can remain refractory to restimulation for long periods of time (see for example Damle et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:5096-5100; Lesslauer et al. (1986) *Eur. J. Immunol.* 16:1289-1295; Gimmi, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88: 6575-6579; Linsley et al. (1991) *J. Exp. Med.* 173:721-730; Koulova et al. (1991) *J. Exp. Med.* 173:759-762; Razi-Wolf, et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4210-4214).
- 25 Alternative to MHC class I antigens, two or more epitopes on other surface antigens on donor cells can be altered. For example, epitopes on MHC class II antigens can be altered. Similar to MHC class I antigens, MHC class II antigens function to present antigenic peptides to the T cell receptor on T lymphocytes. However, MHC class II antigens are present on a limited number of cell types (primarily B cells, macrophages, dendritic cells,
- 30 Langerhans cells and thymic epithelial cells). In addition to or alternative to MHC antigens, epitopes on other antigens on a donor cell which interact with molecules on host T cells and which are known to be involved in immunological rejection of allogeneic or xenogeneic cells can be altered. Other donor cell antigens known to interact with host T cells and to contribute to rejection of a donor cell include molecules which function to increase the
- 35 avidity of the interaction between a donor cell and a host T cell. Due to this property, these molecules are typically referred to as adhesion molecules (although they may serve other functions in addition to increasing the adhesion between a donor cell and a host T cell). Examples of preferred adhesion molecules which can be altered according to the invention include LFA-3, ICAM-1 and ICAM-2. These molecules are ligands for the CD2 and LFA-1

receptors, respectively, on T cells. By altering an adhesion molecule on the donor cell, (such as LFA-3, ICAM-1 or similarly functioning molecule), the ability of the host's T cells to bind to and interact with the donor cell is reduced. Both LFA-3 and ICAM-1 are found on endothelial cells within blood vessels in transplanted organs such as kidney and heart.

- 5 Altering these antigens may facilitate transplantation of any vascularized implant, by preventing recognition of those antigens by CD2+ and LFA-1+ host T-lymphocytes.

The presence of MHC molecules or adhesion molecules such as LFA-3, ICAM-1 etc. on a particular donor cell can be assessed by standard procedures known in the art. For example, the donor cell can be reacted with a labeled antibody directed against the molecule to be detected (e.g., MHC molecule, ICAM-1, LFA-1 etc.) and the association of the labeled  
10 antibody with the cell can be measured by a suitable technique (e.g., immunohistochemistry, flow cytometry etc.).

A preferred method for altering at least two different epitopes on an antigen on a donor cell to inhibit an immune response against the cell is to contact the cell with at least  
15 two different molecules which bind to the epitopes. It is preferred that the cell be contacted with at least two different molecules which bind to the different epitopes prior to administering the cell to a recipient (i.e., the cell is contacted with the molecule *in vitro*). For example, the cell can be incubated with the molecules which bind to the epitopes under conditions which allow binding of the molecules to the epitopes and then any unbound  
20 molecules can be removed (such as described in the Exemplification to follow). Following administration of the donor cell to a recipient, the molecules remain bound to the epitopes on the surface antigen for a sufficient time to interfere with immunological recognition by host cells and induce non-responsiveness in the recipient.

Preferably, the molecule for altering an epitope on a donor cell is an antibody, or  
25 fragment or derivative thereof which retains the ability to bind to the epitope. For use in therapeutic applications, it is necessary that an antibody which binds the epitopes to be altered be unable to fix complement, thus preventing donor cell lysis. Antibody complement fixation can be prevented by deletion of an Fc portion of an antibody, by using an antibody isotype which is not capable of fixing complement, or, less preferably, by using a  
30 complement fixing antibody in conjunction with a drug which inhibits complement fixation. Alternatively, amino acid residues within the Fc region of an antibody which are important for activating complement (see e.g., Tan et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:162-166; Duncan and Winter (1988) *Nature* 332: 738-740) can be mutated to reduce or eliminate the complement-activating ability of an intact antibody. Likewise, amino acids residues within  
35 the Fc region of an antibody which are necessary for binding of the Fc region to Fc receptors (see e.g. Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. et al. (1991) *J. Immunol.* 147:2657-2662) can also be mutated to reduce or eliminate Fc receptor binding if an intact antibody is to be used.

A preferred antibody fragment for altering an epitope is a F(ab')<sub>2</sub> fragment. Antibodies can be fragmented using conventional techniques. For example, the Fc portion of an antibody can be removed by treating an intact antibody with pepsin, thereby generating a F(ab')<sub>2</sub> fragment. In a standard procedure for generating F(ab')<sub>2</sub> fragments, intact antibodies are incubated with immobilized pepsin and the digested antibody mixture is applied to an immobilized protein A column. The free Fc portion binds to the column while the F(ab')<sub>2</sub> fragments pass through the column. The F(ab')<sub>2</sub> fragments can be further purified by HPLC or FPLC. F(ab')<sub>2</sub> fragments can be treated to reduce disulfide bridges to produce Fab' fragments.

10 An antibody, or fragment or derivative thereof, to be used to alter multiple epitopes on an antigen can be derived from polyclonal antisera containing antibodies reactive with a number of epitopes on the antigen. More preferably, however, two different epitopes on the same antigen are altered using two different monoclonal antibodies which bind to two different epitopes on the same antigen (e.g., an MHC class I antigen). Polyclonal and  
15 monoclonal antibodies which bind to different epitopes on one or more antigens can be prepared by standard techniques known in the art. For example, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an antigen (e.g., an MHC class I antigen) or with a cell which expresses the antigen (e.g., on the cell surface) to elicit an antibody response against the antigen in the mammal. Alternatively, tissue or a whole organ which expresses  
20 the antigen can be used to elicit antibodies. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested  
25 from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein ((1975) *Nature* 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., (1983) *Immunol. Today* 4:72), and the EBV-  
30 hybridoma technique to produce human monoclonal antibodies (Cole et al. (1985) *Monoclonal Antibodies in Cancer Therapy*, Allen R. Bliss, Inc., pages 77-96) can be used. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the antigen and monoclonal antibodies isolated.

35 Another method of generating specific antibodies, or antibody fragments, reactive against epitopes on an antigen is to screen expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with the antigen (or a portion thereof). For example, complete Fab fragments, V<sub>H</sub> regions, F<sub>V</sub> regions and single chain antibodies can be expressed in bacteria using phage expression libraries. See for example Ward et al., (1989) *Nature* 341:544-546; Huse et al., (1989) *Science* 246:1275-1281; and McCafferty et

al. (1990) *Nature* 348:552-554. Alternatively, the SCID-hu mouse can be used to produce antibodies, or fragments thereof (available from Genpharm). Antibodies of the appropriate binding specificity which are made by these techniques can be used to alter an antigen on a donor cell.

5       An antibody, or fragment thereof, produced in a non-human subject can be recognized to varying degrees as foreign when the antibody is administered to a human subject (e.g., when a donor cell with an antibody bound thereto is administered to a human subject) and an immune response against the antibody may be generated in the subject. One approach for minimizing or eliminating this problem is to produce chimeric or humanized antibody  
10 derivatives, i.e., antibody molecules comprising portions which are derived from non-human antibodies and portions which are derived from human antibodies. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described. See, for example, Morrison et al., *Proc.*  
15 *Natl. Acad. Sci. U.S.A.* 81, 6851 (1985); Takeda et al., *Nature* 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. For use in therapeutic applications, it is preferred that an antibody used to  
20 used to alter different epitopes on an antigen not contain an Fc portion. Thus, a humanized F(ab')<sub>2</sub> fragment in which parts of the variable region of the antibody, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin is a preferred antibody derivative. Such altered immunoglobulin molecules can be produced by any of several techniques known in the art, (e.g., Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80, 7308-7312 (1983); Kozbor et al.,  
25 *Immunology Today*, 4, 7279 (1983); Olsson et al., *Meth. Enzymol.*, 92, 3-16 (1982)), and are preferably produced according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

30       The ability of two different monoclonal antibodies which bind to the same antigen to bind to different epitopes on the antigen can be determined using a competition binding assay as described in the Exemplification. Briefly, one monoclonal antibody is labeled and used to stain cells which express the antigen. The ability of the unlabeled second monoclonal antibody to inhibit the binding of the first labeled monoclonal antibody to the antigen on the cells is then assessed. If the second monoclonal antibody binds to a different epitope on the  
35 antigen than does the first antibody, the second antibody will be unable to competitively inhibit the binding of the first antibody to the antigen.

Each of the cell surface antigens having two or more epitopes to be altered, e.g., the MHC class I antigens. MHC class II antigens, LFA-3 and ICAM-1 is well-characterized and antibodies reactive with these antigens are commercially available. For example, an antibody

reactive with human MHC class I antigens (i.e., an anti-HLA class I antibody), W6/32, is available from the American Tissue Culture Society (ATCC HB 95). This antibody was raised against human tonsillar lymphocyte membranes and binds to HLA-A, HLA-B and HLA-C (Barnstable, C.J. et al. (1978) *Cell* 14:9-20). Another anti-MHC class I antibody  
5 which can be used is PT85 (see Davis, W.C. et al. (1984) *Hybridoma Technology in Agricultural and Veterinary Research*. N.J. Stern and H.R. Gamble, eds., Rowman and Allenheld Publishers, Totowa, NJ, p121; commercially available from Veterinary Medicine Research Development, Pullman WA). This antibody was raised against swine leukocyte antigens (SLA) and binds to class I antigens from several different species (e.g., pig, human,  
10 mouse, goat). An anti-ICAM-1 antibody can be obtained from AMAC, Inc., Maine. Hybridoma cells producing anti-LFA-3 can be obtained from the American Type Culture Collection, Rockville, Maryland.

Two or more suitable antibodies, or fragments or derivatives thereof, for use in the invention can be identified based upon their ability to inhibit an immunological rejection of  
15 allogeneic or xenogeneic cells. Briefly, the antibodies (or antibody fragments) are incubated for a short period of time (e.g., 30 minutes at room temperature) with cells or tissue to be transplanted, any unbound antibody is washed away. The cells or tissue are then transplanted into a recipient animal. The ability of the multiple antibody pretreatment to inhibit or prevent rejection of the transplanted cells or tissue is then determined by monitoring the function of  
20 the graft and/or by monitoring for signs of rejection of the cells or tissue compared to untreated controls.

Other molecules which bind to an epitope on an antigen on a donor cell and produce a functionally similar result as antibodies, or fragments or derivatives thereof, (e.g., other molecules which interfere with the interaction of the antigen with a hematopoietic cell and  
25 induce immunological nonresponsiveness) can be used to alter the epitope on the donor cell. One such molecule is a soluble form of a ligand for an antigen (e.g., a receptor) on the donor cell which could be used to alter an epitope on the antigen on the donor cell. For example, a soluble form of CD2 (i.e., comprising the extracellular domain of CD2 without the transmembrane or cytoplasmic domain) can be used to alter an epitope on LFA-3 on the  
30 donor cell by binding to LFA-3 on donor cells in a manner analogous to an antibody. Alternatively, a soluble form of LFA-1 can be used to alter an epitope on ICAM-1 on the donor cell. A soluble form of a ligand can be made by standard recombinant DNA procedures, using a recombinant expression vector containing DNA encoding the ligand encompassing only the extracellular domain (i.e., lacking DNA encoding the transmembrane and cytoplasmic domains). The recombinant expression vector encoding the extracellular  
35 domain of the ligand can be introduced into host cells to produce a soluble ligand, which can then be isolated. Soluble ligands of use have a binding affinity for the receptor on the donor cell sufficient to remain bound to the receptor to interfere with immunological recognition and induce non-responsiveness when the cell is administered to a recipient (e.g., preferably,

the affinity for binding of the soluble ligand to the receptor is at least about  $10^{-7}$  M). Additionally, the soluble ligand can be in the form of a fusion protein comprising the receptor binding portion of the ligand fused to another protein or portion of a protein. For example, an immunoglobulin fusion protein which includes an extracellular domain, or functional portion of CD2 or LFA-1 linked to an immunoglobulin heavy chain constant region (e.g., the hinge, CH2 and CH3 regions of a human immunoglobulin such as IgG1) can be used. Immunoglobulin fusion proteins can be prepared, for example, according to the teachings of Capon, D.J. et al. (1989) *Nature* 337:525-531 and U.S. Patent No. 5,116,964 to Capon and Lasky.

Another type of molecule which can be used to alter an MHC antigen (e.g., and MHC class I antigen) is a peptide which binds to the MHC antigen and interferes with the interaction of the MHC antigen with a T lymphocyte. In one embodiment, the soluble peptide mimics a region of the T cell receptor which contacts the MHC antigen. This peptide can be used to interfere with the interaction of the intact T cell receptor (on a T lymphocyte) with the MHC antigen. Such a peptide binds to a region of the MHC molecule which is specifically recognized by a portion of the T cell receptor (e.g., the alpha-1 or alpha-2 loop of an MHC class I antigen), thereby altering the MHC class I antigen and inhibiting recognition of the antigen by the T cell receptor. In another embodiment, the soluble peptide mimics a region of a T cell surface molecule which contacts the MHC antigen, such as a region of the CD8 molecule which contacts an MHC class I antigen or a region of a CD4 molecule which contacts an MHC class II antigen. For example, a peptide which binds to a region of the alpha-3 loop of an MHC class I antigen can be used to inhibit binding to CD8 to the antigen, thereby inhibiting recognition of the antigen by T cells. T cell receptor-derived peptides have been used to inhibit MHC class I-restricted immune responses (see e.g., Clayberger, C. et al. (1993) *Transplant Proc.* 25:477-478) and prolong allogeneic skin graft survival *in vivo* when injected subcutaneously into the recipient (see e.g., Goss, J.A. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:9872-9876).

It is preferred that an antibody, or fragment or derivative thereof, which is used to alter an epitope have an affinity for binding to its target epitope of at least  $10^{-7}$  M. The affinity of an antibody or other molecule for binding to an epitope on an antigen can be determined by conventional techniques (see Masan, D.W. and Williams, A.F. (1980) *Biochem. J.* 187:1-10). Briefly, the antibody to be tested is labeled with  $I^{125}$  and incubated with cells expressing the antigen at increasing concentrations until equilibrium is reached. Data are plotted graphically as [bound antibody]/[free antibody] versus [bound antibody] and the slope of the line is equal to the  $K_D$  (Scatchard analysis).

The same or different types of molecules can be used to alter two or more different epitopes on a donor cell. In a preferred embodiment, two different antibodies (or fragments thereof) are used to alter two different epitopes. Alternatively, one epitope can be altered with one type of molecule and a second epitope can be altered with another type of molecule. For

example, two different epitopes on the same MHC class I antigen can be altered using an anti-MHC class I antibody and an MHC-binding peptide.

Alternative to binding one or more molecules (e.g., an antibodies) to epitopes on an antigen on a donor cell to inhibit immunological rejection of the cell, the epitopes can be altered by other means. For example, epitopes can be directly altered (e.g., mutated) such that they can no longer interact normally with a hematopoietic cell (e.g., a T lymphocyte) in an allogeneic or xenogeneic recipient and induces immunological non-responsiveness to the donor cell in the recipient. For example, an altered form of an MHC class I antigen or adhesion molecule (e.g., LFA-3 or ICAM-1), in which two or more epitopes are mutated, can be created by mutagenesis and selected in *in vitro* culture based upon the failure of the molecule to contribute to T cell activation. An altered form of an MHC class I antigen or adhesion molecule delivers an inappropriate or insufficient signal to a T cell upon binding to a receptor on the T cell. A nucleic acid encoding the mutated form of the antigen (i.e., the antigen with mutated epitopes) can then be inserted into the genome of a non-human animal, either as a transgene or by homologous recombination (to replace the endogenous gene encoding the wild-type antigen). Cells from the non-human animal which express the mutated form of the antigen can then be used as a donor cell for transplantation into an allogeneic or xenogeneic recipient.

A cell of the invention which has a natural antibody epitope altered, reduced or substantially eliminated can additionally be modified to express a gene product, such as a gene product to be provided to a recipient for therapeutic purposes. The gene product can be, for example, a secreted protein, a membrane-bound protein or an intracellular protein. Other gene products include active RNA molecules. Non-limiting examples of secreted gene products of therapeutic interest which a cell of the invention can be modified to express include  $\alpha$ 1-antitrypsin, apoA1, TNF, soluble TNF receptor, human growth hormone, insulin, erythropoietin, anti-angiogenesis factors and interleukins. For example, the secreted protein can replace a missing function in a subject (e.g., insulin in a diabetic subject) or can stimulate a response in a subject (e.g., TNF or IL-2 can be produced in a tumor-bearing subject to stimulate an immune response against the tumor in the subject). Alternatively, the gene product can be a membrane-bound protein. In this case, the gene product remains associated with the membrane of the modified donor cell and functions, for example, by binding a soluble substance in a host (e.g., binding of LDL cholesterol by an LDL receptor) or by binding to another membrane-bound protein (e.g., a receptor) on cells of the host to trigger a signal within the recipient cells. Non-limiting examples of membrane-bound gene products which a cell can be modified to express include the LDL receptor, CFTR and CD18. Alternatively, the gene product can be an intracellular protein. The intracellular protein within modified donor cells can be introduced into cells of a recipient by fusion of the donor cells to recipient cells (e.g., fusion of modified myoblasts or myocytes with muscle cells within the recipient, e.g., to deliver dystrophin). An intracellular protein can also function by

acting upon substances within a recipient that are taken up by the modified cell (e.g., to detoxify substances within the recipient). Non-limiting examples of intracellular proteins which a cell can be modified to express include glucocerebrosidase,  $\beta$ -glucuronidase, dystrophin,  $\beta$ -globin, phenylalanine hydroxylase, tyrosine hydroxylase, ornithine

5 transcarbamylase, arginosuccinate synthetase, UDP-glucuronosyl transferase and adenosine deaminase. Preferably, a cell is modified to express a gene product by introducing into the cell a nucleic acid encoding the gene product in a form suitable for expression of the gene product in the cell. For example, a recombinant expression vector (e.g., a viral vector) containing a gene of interest can be prepared and introduced into a cell of the invention by

10 methods described above regarding antisense expression vectors (or by other conventional techniques).

As used herein, the term "modified to express a gene product" is intended to include a cell treated in a manner that results in the production of a gene product by the cell. Preferably, the cell does not express the gene product prior to modification. Alternatively,

15 modification of the cell may result in an increased production of a gene product already expressed by the cell or result in production of a gene product (e.g., an antisense RNA molecule) which decreases production of another, undesirable gene product normally expressed by the cell.

In a preferred embodiment, a cell is modified to express a gene product by

20 introducing genetic material, such as a nucleic acid molecule (e.g., RNA or, more preferably, DNA) into the cell. The nucleic acid molecule introduced into the cell encodes a gene product to be expressed by the cell. The term "gene product" as used herein is intended to include proteins, peptides and functional RNA molecules. Generally, the gene product encoded by the nucleic acid molecule is the desired gene product to be supplied to a subject.

25 Alternatively, the encoded gene product is one which induces the expression of the desired gene product by the cell (e.g., the introduced genetic material encodes a transcription factor which induces the transcription of the gene product to be supplied to the subject).

A nucleic acid molecule introduced into a cell is in a form suitable for expression in the cell of the gene product encoded by the nucleic acid. Accordingly, the nucleic acid

30 molecule includes coding and regulatory sequences required for transcription of a gene (or portion thereof) and, when the gene product is a protein or peptide, translation of the gene product encoded by the gene. Regulatory sequences which can be included in the nucleic acid molecule include promoters, enhancers and polyadenylation signals, as well as sequences necessary for transport of an encoded protein or peptide, for example N-terminal

35 signal sequences for transport of proteins or peptides to the surface of the cell or for secretion.

Nucleotide sequences which regulate expression of a gene product (e.g., promoter and enhancer sequences) are selected based upon the type of cell in which the gene product is to be expressed and the desired level of expression of the gene product. For example, a promoter known to confer cell-type specific expression of a gene linked to the promoter can



be used. A promoter specific for myoblast gene expression can be linked to a gene of interest to confer muscle-specific expression of that gene product. Muscle-specific regulatory elements which are known in the art include upstream regions from the dystrophin gene (Klamut et al., (1989) *Mol. Cell. Biol.* 9:2396), the creatine kinase gene (Buskin and Hauschka, (1989) *Mol. Cell Biol.* 9:2627) and the troponin gene (Mar and Ordahl, (1988) *Proc. Natl. Acad. Sci. USA.* 85:6404). Regulatory elements specific for other cell types are known in the art (e.g., the albumin enhancer for liver-specific expression; insulin regulatory elements for pancreatic islet cell-specific expression; various neural cell-specific regulatory elements, including neural dystrophin, neural enolase and A4 amyloid promoters).

Alternatively, a regulatory element which can direct constitutive expression of a gene in a variety of different cell types, such as a viral regulatory element, can be used. Examples of viral promoters commonly used to drive gene expression include those derived from polyoma virus, Adenovirus 2, cytomegalovirus and Simian Virus 40, and retroviral LTRs.

Alternatively, a regulatory element which provides inducible expression of a gene linked thereto can be used. The use of an inducible regulatory element (e.g., an inducible promoter) allows for modulation of the production of the gene product in the cell. Examples of potentially useful inducible regulatory systems for use in eukaryotic cells include hormone-regulated elements (e.g., see Mader, S. and White, J.H. (1993) *Proc. Natl. Acad. Sci. USA* 90:5603-5607), synthetic ligand-regulated elements (see, e.g. Spencer, D.M. et al. (1993) *Science* 262:1019-1024) and ionizing radiation-regulated elements (e.g., see Manome, Y. et al. (1993) *Biochemistry* 32:10607-10613; Datta, R. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10149-10153). Additional tissue-specific or inducible regulatory systems which may be developed can also be used in accordance with the invention.

There are a number of techniques known in the art for introducing genetic material into a cell that can be applied to modify a cell of the invention. In one embodiment, the nucleic acid is in the form of a naked nucleic acid molecule. In this situation, the nucleic acid molecule introduced into a cell to be modified consists only of the nucleic acid encoding the gene product and the necessary regulatory elements. Alternatively, the nucleic acid encoding the gene product (including the necessary regulatory elements) is contained within a plasmid vector. Examples of plasmid expression vectors include CDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman, et al., *EMBO J.* 6:187-195 (1987)). In another embodiment, the nucleic acid molecule to be introduced into a cell is contained within a viral vector. In this situation, the nucleic acid encoding the gene product is inserted into the viral genome (or a partial viral genome). The regulatory elements directing the expression of the gene product can be included with the nucleic acid inserted into the viral genome (i.e. linked to the gene inserted into the viral genome) or can be provided by the viral genome itself. Examples of methods which can be used to introduce naked nucleic acid into cells and viral-mediated transfer of nucleic acid into cells are described separately in the subsections below.

#### A. Introduction of Naked Nucleic Acid into Cells

1. *Transfection mediated by CaPO<sub>4</sub>*: Naked DNA can be introduced into cells by forming a precipitate containing the DNA and calcium phosphate. For example, a HEPES-buffered saline solution can be mixed with a solution containing calcium chloride and DNA to form a precipitate and the precipitate is then incubated with cells. A glycerol or dimethyl sulfoxide shock step can be added to increase the amount of DNA taken up by certain cells. CaPO<sub>4</sub>-mediated transfection can be used to stably (or transiently) transfect cells and is only applicable to *in vitro* modification of cells. Protocols for CaPO<sub>4</sub>-mediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.1 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.32-16.40 or other standard laboratory manuals.
2. *Transfection mediated by DEAE-dextran*: Naked DNA can be introduced into cells by forming a mixture of the DNA and DEAE-dextran and incubating the mixture with the cells. A dimethylsulfoxide or chloroquine shock step can be added to increase the amount of DNA uptake. DEAE-dextran transfection is only applicable to *in vitro* modification of cells and can be used to introduce DNA transiently into cells but is not preferred for creating stably transfected cells. Thus, this method can be used for short term production of a gene product but is not a method of choice for long-term production of a gene product. Protocols for DEAE-dextran-mediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.2 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.41-16.46 or other standard laboratory manuals.
3. *Electroporation*: Naked DNA can also be introduced into cells by incubating the cells and the DNA together in an appropriate buffer and subjecting the cells to a high-voltage electric pulse. The efficiency with which DNA is introduced into cells by electroporation is influenced by the strength of the applied field, the length of the electric pulse, the temperature, the conformation and concentration of the DNA and the ionic composition of the media. Electroporation can be used to stably (or transiently) transfect a wide variety of cell types and is only applicable to *in vitro* modification of cells. Protocols for electroporating cells can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.3 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.54-16.55 or other standard laboratory manuals.
4. *Liposome-mediated transfection ("lipofection")*: Naked DNA can be introduced into cells by mixing the DNA with a liposome suspension containing cationic lipids. The

DNA/liposome complex is then incubated with cells. Liposome mediated transfection can be used to stably (or transiently) transfect cells in culture *in vitro*. Protocols can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.4 and other standard laboratory manuals. Additionally, gene delivery *in vivo* has been accomplished using liposomes. See for example Nicolau et al. (1987) *Meth. Enz.* 149:157-176; Wang and Huang (1987) *Proc. Natl. Acad. Sci. USA* 84:7851-7855; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278; and Gould-Fogerite et al. (1989) *Gene* 84:429-438.

10 5. *Direct Injection*: Naked DNA can be introduced into cells by directly injecting the DNA into the cells. For an *in vitro* culture of cells, DNA can be introduced by microinjection. Since each cell is microinjected individually, this approach is very labor intensive when modifying large numbers of cells. However, a situation wherein microinjection is a method of choice is in the production of transgenic animals (discussed in greater detail below). In this situation, the DNA is stably introduced into a fertilized oocyte which is then allowed to develop into an animal. The resultant animal contains cells carrying the DNA introduced into the oocyte. Direct injection has also been used to introduce naked DNA into cells *in vivo* (see e.g., Acsadi et al. (1991) *Nature* 332: 815-818; Wolff et al. (1990) *Science* 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* can be used. 20 Such an apparatus is commercially available (e.g., from BioRad).

6. *Receptor-Mediated DNA Uptake*: Naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; 25 Wilson et al. (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. Receptors to which a DNA-ligand complex have targeted include the transferrin receptor and the asialoglycoprotein receptor. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example 30 Curiel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126). Receptor-mediated DNA uptake can be used to introduce DNA into cells either *in vitro* or *in vivo* and, additionally, has the added feature that DNA can be selectively targeted to a particular cell type by use of a ligand which binds to a receptor 35 selectively expressed on a target cell of interest.

Generally, when naked DNA is introduced into cells in culture (e.g., by one of the transfection techniques described above) only a small fraction of cells (about 1 out of  $10^5$ ) typically integrate the transfected DNA into their genomes (i.e., the DNA is maintained in the cell episomally). Thus, in order to identify cells which have taken up exogenous DNA, it is

advantageous to transfect nucleic acid encoding a selectable marker into the cell along with the nucleic acid(s) of interest. Preferred selectable markers include those which confer resistance to drugs such as G418, hygromycin and methotrexate. Selectable markers may be introduced on the same plasmid as the gene(s) of interest or may be introduced on a separate  
5 plasmid.

An alternative method for generating a cell that is modified to express a gene product involving introducing naked DNA into cells is to create a transgenic animal which contains cells modified to express the gene product of interest. A transgenic animal is an animal having cells that contain a transgene, wherein the transgene was introduced into the animal or  
10 an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA molecule which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. Thus, a transgenic animal expressing a gene product of interest in one or more cell  
15 types within the animal can be created, for example, by introducing a nucleic acid encoding the gene product (typically linked to appropriate regulatory elements, such as a tissue-specific enhancer) into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art  
20 and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan, B. et al., (1986) *A Laboratory Manual*, Cold Spring Harbor, New York, Cold Spring Harbor Laboratory. A transgenic founder animal can be used to breed more animals carrying the transgene. Cells of the transgenic animal which express a gene product of interest can then be used to deliver the gene product to a subject in accordance with the invention.

25 Alternatively, an animal containing a gene which has been modified by homologous recombination can be constructed to express a gene product of interest. For example, an endogenous gene carried in the genome of the animal can be altered by homologous recombination (for instance, all or a portion of a gene could be replaced by the human homologue of the gene to "humanize" the gene product encoded by the gene) or an  
30 endogenous gene can be "knocked out" (i.e., inactivated by mutation). For example, an endogenous gene in a cell can be knocked out to prevent production of that gene product and then nucleic acid encoding a different (preferred) gene product is introduced into the cell. To create an animal with homologously recombined nucleic acid, a vector is prepared which contains the DNA which is to replace or interrupt the endogenous DNA flanked by DNA  
35 homologous to the endogenous DNA (see for example Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503). The vector is introduced into an embryonal stem cell line (e.g., by electroporation) and cells which have homologously recombined the DNA are selected (see for example Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see for example

Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term.

- Progeny harbouring the homologously recombined DNA in their germ cells can be used to  
 5 breed animals in which all cells of the animal contain the homologously recombined DNA. Cells of the animal containing the homologously recombined DNA which express a gene product of interest can then be used to deliver the gene product to a subject in accordance with the invention.

## 10 B. Viral-Mediated Gene Transfer

- A preferred approach for introducing nucleic acid encoding a gene product into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the  
 15 nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid and viral vector systems can be used either *in vitro* or *in vivo*.

1. *Retroviruses*: Defective retroviruses are well characterized for use in gene transfer for  
 20 gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene product of interest inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper  
 25 virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art.
- 30 Examples of suitable packaging virus lines include  $\psi$ Crip,  $\psi$ Cre,  $\psi$ 2 and  $\psi$ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl.*  
 35 *Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu

et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

2. *Adenoviruses*: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*), endothelial cells (Lemarchand et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

3. *Adeno-Associated Viruses*: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types

using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

5           The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (e.g., Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase  
10 chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product, such as an enzymatic assay. If the gene product of interest to be expressed by a cell is not readily assayable, an expression system can first be optimized using a reporter gene linked to the  
15 regulatory elements and vector to be used. The reporter gene encodes a gene product which is easily detectable and, thus, can be used to evaluate the efficacy of the system. Standard reporter genes used in the art include genes encoding  $\beta$ -galactosidase, chloramphenicol acetyl transferase, luciferase and human growth hormone.

          When the method used to introduce nucleic acid into a population of cells results in  
20 modification of a large proportion of the cells and efficient expression of the gene product by the cells (e.g., as is often the case when using a viral expression vector), the modified population of cells may be used without further isolation or subcloning of individual cells within the population. That is, there may be sufficient production of the gene product by the population of cells such that no further cell isolation is needed. Alternatively, it may be  
25 desirable to grow a homogenous population of identically modified cells from a single modified cell to isolate cells which efficiently express the gene product. Such a population of uniform cells can be prepared by isolating a single modified cell by limiting dilution cloning followed by expanding the single cell in culture into a clonal population of cells by standard techniques.

30           Alternative to introducing a nucleic acid molecule into a cell to modify the cell to express a gene product, a cell can be modified by inducing or increasing the level of expression of the gene product by a cell. For example, a cell may be capable of expressing a particular gene product but fails to do so without additional treatment of the cell. Similarly, the cell may express insufficient amounts of the gene product for the desired purpose. Thus,  
35 an agent which stimulates expression of a gene product can be used to induce or increase expression of a gene product by the cell. For example, cells can be contacted with an agent *in vitro* in a culture medium. The agent which stimulates expression of a gene product may function, for instance, by increasing transcription of the gene encoding the product, by increasing the rate of translation or stability (e.g., a post transcriptional modification such as a

poly A tail) of an mRNA encoding the product or by increasing stability, transport or localization of the gene product. Examples of agents which can be used to induce expression of a gene product include cytokines and growth factors.

Another type of agent which can be used to induce or increase expression of a gene product by a cell is a transcription factor which upregulates transcription of the gene encoding the product. A transcription factor which upregulates the expression of a gene encoding a gene product of interest can be provided to a cell, for example, by introducing into the cell a nucleic acid molecule encoding the transcription factor. Thus, this approach represents an alternative type of nucleic acid molecule which can be introduced into the cell (for example by one of the previously discussed methods). In this case, the introduced nucleic acid does not directly encode the gene product of interest but rather causes production of the gene product by the cell indirectly by inducing expression of the gene product.

In yet another method, a cell is modified to express a gene product by coupling the gene product to the cell, preferably to the surface of the cell. For example, a protein can be obtained by purifying the cell from a biological source or expressing the protein recombinantly using standard recombinant DNA technology. The isolated protein can then be coupled to the cell. The terms "coupled" or "coupling" refer to a chemical, enzymatic or other means (e.g., by binding to an antibody on the surface of the cell or genetic engineering of linkages) by which a gene product can be linked to a cell such that the gene product is in a form suitable for delivering the gene product to a subject. For example, a protein can be chemically crosslinked to a cell surface using commercially available crosslinking reagents (Pierce, Rockford IL). Other approaches to coupling a gene product to a cell include the use of a bispecific antibody which binds both the gene product and a cell-surface molecule on the cell or modification of the gene product to include a lipophilic tail (e.g., by inositol phosphate linkage) which can insert into a cell membrane.

#### IV. Methods of the Invention

Another aspect of the invention pertains to methods for reducing the immunogenicity of a cell for transplantation. A cell for use in this method is one which has at least one epitope on its surface which stimulates hyperacute rejection of the cell by natural antibodies in a recipient subject. The immunogenicity of the cell is reduced by contacting the cell with an agent which alters, reduces or substantially eliminates expression of the epitope on the cell surface, thereby reducing the capacity of the cell to stimulate natural antibody-mediated hyperacute rejection of the cell in a recipient. Preferably, the epitope is a carbohydrate, such as a galactosyl( $\alpha$ 1-3)galactose epitope.

An epitope on the surface of the cell is altered, reduced or substantially eliminated by one of the treatments previously described in Section II. Accordingly, in one embodiment, the agent is one which cleaves the epitope from the cell surface, such as an enzyme (e.g., an  $\alpha$ -galactosidase) or a chemical. In another embodiment, the agent is one which inhibits



the formation of the epitope on the cell surface, for example by inhibiting the activity of a glycosyltransferase in the cell (e.g., an  $\alpha$ 1-3-galactosyltransferase). Thus, the agent can be an antisense nucleic acid or a chemical inhibitor of the enzyme. In yet another embodiment, the agent is one which binds to the epitope and inhibits binding of natural antibodies to the epitope in a recipient, such as a lectin or an antibody (or fragment thereof) which does not activate complement or cause lysis of the cell.

An epitope on a cell surface can be altered, reduced or substantially eliminated *in vitro* or *in vivo*. Accordingly, the term "contacting" is intended to encompass either incubating a cell with the agent *in vitro* or administering the agent to a subject (e.g., a transplant recipient). Alternatively, a cell can be treated *in vitro*, administered to a subject and then further treated *in vivo* in the subject (e.g., a cell to be transplanted is incubated *in vitro* with antisense oligonucleotides, the cells are administered to a subject and then additional antisense oligonucleotides are administered to the subject; see Example 4). The agent is contacted with the cell in an amount and for a period of time sufficient to alter, reduce or substantially eliminate expression of the epitope such that hyperacute rejection of the cell is inhibited in a recipient.

After a cell is treated *in vitro* to alter, reduce or substantially eliminate the expression of at least one natural antibody epitope on the cell surface, the cell is administered to a recipient. Accordingly, another aspect of the invention pertains to methods for transplanting a cell into a recipient subject such that hyperacute rejection of the cell by the subject is inhibited. The term "subject" is intended to include humans and nonhuman primates (e.g., Old World Monkeys). The method involves contacting the cell, prior to transplantation, with an agent which alters, reduces or substantially eliminates expression of at least one epitope on the cell surface which stimulates hyperacute rejection of the cell in the subject and then administering the cell to the subject. Agents which are used to alter, reduce or substantially eliminate expression of the epitope are as described above. The cell is administered to the subject in an amount and by a route which is suitable for the desired therapeutic result. The cell used in the method can be within a tissue or organ. Accordingly, in these embodiments, the tissue or organ is transplanted into the recipient by conventional techniques for transplantation. Acceptance of transplanted cells, tissues or organs can be determined morphologically (e.g., with skin grafts by examining the transplanted tissue or by biopsy) or by assessment of the functional activity of the graft. For example, acceptance of pancreatic islet cells can be determined by measuring insulin production, acceptance of liver cells can be determined by assessing albumin production and acceptance of neural cells can be determined by assessing neural cell function.

In addition to treatment of a cell to be transplanted to reduce hyperacute rejection of the cell in a recipient, the method for transplantation of the invention can include additional *in vitro* treatment of the cells prior to transplantation and/or additional *in vivo* treatment of the recipient following transplantation to further inhibit immunological rejection of the

transplanted cells. For example, prior to transplantation, the cell can be contacted with a second agent which alters expression of at least one antigen on the cell surface which is capable of stimulating a cellular immune response against the cell in the subject. Antigens to be altered, and methods for alteration of the antigen, are as described in Section III above.

- 5 Preferably, an MHC class I antigen is altered on the cell surface by contacting the cell prior to transplantation with an anti-MHC class I antibody, or fragment thereof (e.g., an anti-MHC class I F(ab')<sub>2</sub> fragment).

Additionally or alternatively, a recipient subject can be treated prior to, during and/or following transplantation with another agent which inhibits T cell activity in the subject.

- 10 Thus, in one embodiment, a cell to be transplanted is treated with a first agent which alters, reduces or substantially eliminates expression of a cell surface natural antibody epitope and the transplant recipient is treated with a second agent which inhibits T cell activity. In another embodiment, a cell to be transplanted is treated both with a first agent which alters, reduces or substantially eliminates expression of a cell surface natural antibody epitope and
- 15 with a second agent which alters an antigen on the cell surface which stimulates a cellular immune response against the cell, and the transplant recipient is treated with a third agent which inhibits T cell activity. As used herein, an agent which inhibits T cell activity is defined as an agent which results in removal (e.g., sequestration) or destruction of T cells within a subject or inhibits T cell functions within the subject (i.e., T cells may still be
- 20 present in the subject but are in a non-functional state, such that they are unable to proliferate or elicit or perform effector functions, e.g. cytokine production, cytotoxicity etc.). The term "T cell" encompasses mature peripheral blood T cells lymphocytes. The agent which inhibits T cell activity may also inhibit the activity or maturation of immature T cells (e.g., thymocytes).

- 25 The agent which inhibits T cell activity in a subject can be an immunosuppressive drug. The term "immunosuppressive drug" is intended to include pharmaceutical agents which inhibit or interfere with normal immune function. A preferred immunosuppressive drug is cyclosporin A. Other immunosuppressive drugs which can be used include FK506 and RS-61443. In one embodiment, the immunosuppressive drug is administered in
- 30 conjunction with at least one other therapeutic agent. Additional therapeutic agents which can be administered include steroids (e.g., glucocorticoids such as prednisone, methyl prednisolone and dexamethasone) and chemotherapeutic agents (e.g., azathioprine and cyclophosphamide). In another embodiment, an immunosuppressive drug is administered in conjunction with both a steroid and a chemotherapeutic agent. Suitable immunosuppressive
- 35 drugs are commercially available (e.g., cyclosporin A is available from Sandoz, Corp., East Hanover, NJ).

An immunosuppressive drug is administered in a formulation which is compatible with the route of administration. Suitable routes of administration include intravenous injection (either as a single infusion, multiple infusions or as an intravenous drip over time).

intraperitoneal injection, intramuscular injection and oral administration. For intravenous injection, the drug can be dissolved in a physiologically acceptable carrier or diluent (e.g., a buffered saline solution) which is sterile and allows for syringability. Dispersions of drugs can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils.

5 Convenient routes of administration and carriers for immunosuppressive drugs are known in the art. For example, cyclosporin A can be administered intravenously in a saline solution, or orally, intraperitoneally or intramuscularly in olive oil or other suitable carrier or diluent.

An immunosuppressive drug is administered to a recipient subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of transplanted

10 cells). Dosage ranges for immunosuppressive drugs, and other agents which can be coadministered therewith (e.g., steroids and chemotherapeutic agents), are known in the art (see e.g., Freed et al. (1992) *New Engl. J. Med.* 327:1549; Spencer et al. (1992) *New Engl. J. Med.* 327:1541; Widner et al. (1992) *New Engl. J. Med.* 327:1556; Lindvall et al. (1992) *Ann. Neurol.* 31:155; and Lindvall et al. (1992) *Arch. Neurol.* 46:615). A preferred dosage range

15 for immunosuppressive drugs, suitable for treatment of humans, is about 1-30 mg/kg of body weight per day. A preferred dosage range for cyclosporin A is about 1-10 mg/kg of body weight per day, more preferably about 1-5 mg/kg of body weight per day. Dosages can be adjusted to maintain an optimal level of the immunosuppressive drug in the serum of the recipient subject. For example, dosages can be adjusted to maintain a preferred serum level

20 for cyclosporin A in a human subject of about 100-200 ng/ml. It is to be noted that dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the dosage

25 ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

In one embodiment of the invention, an immunosuppressive drug is administered to a subject transiently for a sufficient time to induce T cell tolerance to the transplanted cells in the subject. Transient administration of an immunosuppressive drug has been found to

30 induce long-term graft-specific tolerance in a graft recipient (see Brunson et al. (1991) *Transplantation* 52:545; Hutchinson et al. (1981) *Transplantation* 32:210; Green et al. (1979) *Lancet* 2:123; Hall et al. (1985) *J. Exp. Med.* 162:1683). Administration of the drug to the subject can begin prior to transplantation of the cells into the subject. For example, initiation of drug administration can be a few days (e.g., one to three days) before transplantation.

35 Alternatively, drug administration can begin the day of transplantation or a few days (generally not more than three days) after transplantation. Administration of the drug is continued for sufficient time to induce donor cell-specific tolerance in the recipient such that donor cells will continue to be accepted by the recipient when drug administration ceases. For example, the drug can be administered for as short as three days or as long as three

months following transplantation. Typically, the drug is administered for at least one week but not more than one month following transplantation. Induction of tolerance to the transplanted cells in a subject is indicated by the continued acceptance of the transplanted cells after administration of the immunosuppressive drug has ceased. Acceptance of transplanted tissue can be determined morphologically or functionally, as described above.

Another type of agent which can be used to inhibit T cell activity in a subject is an antibody, or fragment or derivative thereof, which depletes or sequesters T cells in a recipient. Antibodies which are capable of depleting or sequestering T cells *in vivo* when administered to a subject are known in the art. Typically, these antibodies bind to an antigen on the surface of a T cell. Polyclonal antisera can be used, for example anti-lymphocyte serum. Alternatively, one or more monoclonal antibodies can be used. Preferred T cell-depleting antibodies include monoclonal antibodies which bind to CD2, CD3, CD4 or CD8 on the surface of T cells. Antibodies which bind to these antigens are known in the art and are available (e.g., from American Tissue Type Collection). A preferred monoclonal antibody for binding to CD3 on human T cells is OKT3 (ATCC CRL 8001). The binding of an antibody to surface antigens on a T cell can facilitate sequestration of T cells in a subject and/or destruction of T cells in a subject by endogenous mechanisms. Alternatively, a T cell-depleting antibody which binds to an antigen on a T cell surface can be conjugated to a toxin (e.g., ricin) or other cytotoxic molecule (e.g., a radioactive isotope) to facilitate destruction of T cells upon binding of the antibody to the T cells.

Another type of antibody which can be used to inhibit T cell activity in a recipient subject is an antibody which inhibits T cell proliferation. For example, an antibody directed against a T cell growth factor, such as IL-2, or a T cell growth factor receptor, such as the IL-2 receptor, can inhibit proliferation of T cells (see e.g., DeSilva, D.R. et al. (1991) *J. Immunol.* 147:3261-3267). Accordingly, an anti-IL-2 or an anti-IL-2 receptor antibody can be administered to a recipient to inhibit rejection of a transplanted cell (see e.g. Wood et al. (1992) *Neuroscience* 49:410). Additionally, both an anti-IL-2 and an anti-IL-2 receptor antibody can be coadministered to inhibit T cell activity or can be administered with another antibody (e.g., which binds to a surface antigen on T cells).

An antibody which depletes, sequesters or inhibits T cells within a recipient can be administered at a dose and for an appropriate time to inhibit rejection of cells upon transplantation. Antibodies are preferably administered intravenously in a pharmaceutically acceptable carrier or diluent (e.g., a sterile saline solution). Antibody administration can begin prior to transplantation (e.g., one to five days prior to transplantation) and can continue on a daily basis after transplantation to achieve the desired effect (e.g., up to fourteen days after transplantation). A preferred dosage range for administration of an antibody to a human subject is about 0.1-0.3 mg/kg of body weight per day. Alternatively, a single high dose of antibody (e.g., a bolus at a dosage of about 10 mg/kg of body weight) can be administered to a human subject on the day of transplantation. The effectiveness of antibody treatment in

depleting T cells from the peripheral blood can be determined by comparing T cell counts in blood samples taken from the subject before and after antibody treatment. Dosage regimens may be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

This invention is further illustrated by the following Examples which should not be construed as limiting. The contents of all references and published patents and patent applications cited throughout the application are hereby incorporated by reference.

10

**EXAMPLE 1: Removal of Galactosyl( $\alpha$ 1,3)Galactose Epitopes from Endothelial Cells by Alpha-Galactosidase Treatment**

In this example, porcine endothelial cells were treated with the enzyme alpha-galactosidase to remove terminal galactosyl( $\alpha$ 1,3)galactose epitopes on the cell surface which are recognized by natural antibodies present in sera from humans and other primates. However, prior to treating the cells with alpha-galactosidase, the following experiment was performed to determine whether human and other primate (e.g., monkey) serum contained antibodies that reacted with porcine endothelial cells.

Porcine endothelial cells were isolated from a swine aorta by collagenase digestion and cloned by limiting dilution in DMEM (high glucose; commercially obtained from Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Intergen, Purchase, NY) and 1% penicillin/streptomycin (BioWitaker, Walkersville, MD). After isolation, the cells were incubated at 37°C with 5% CO<sub>2</sub>. The cells were harvested from culture by trypsinization (trypsin obtained from BioWitaker, Walkersville, MD), neutralized with culture medium, washed twice with PBS and resuspended in PBS/0.5% bovine serum albumin (BSA). Fifty  $\mu$ l (1x10<sup>5</sup> cells) were mixed with 50  $\mu$ l of 1:10 diluted human or cynomolgus monkey serum (New England Regional Primate Research Center, Southborough, MA) and incubated on ice for 1 hour. After the primary incubation, cells were washed 3 times with PBS/0.5% BSA and 50  $\mu$ l of a 1:50 dilution of the following fluorescein conjugated antibodies were added to the appropriate tubes: goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA), goat anti-human IgM (Jackson ImmunoResearch), rabbit anti-monkey IgG (Accurate Chemical, Westbury, NY), goat anti-monkey IgM (Nordic, Netherlands) and GS-I B4 (EY Laboratories, San Mateo, CA). For detection of swine lymphocyte antigen (SLA) the monoclonal antibody PT-85 (VMRD, Pullman, WA) was employed with a goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch). The cells were incubated with secondary antibody for 30 minutes on ice. After incubation, the tubes were washed 3 times with PBS/0.5% BSA and resuspended in 500  $\mu$ l PBS/0.5% BSA. All samples were analyzed by flow cytometry at 488 nm. The results are illustrated in Figures 1A-1D. The data indicate that

antibodies in monkey and human serum recognize determinants present on porcine endothelial cells. Both IgG and IgM antibodies displayed strong reactivity with porcine endothelial cells when detected with anti-human or anti-monkey IgG or IgM secondary antibodies.

5 To identify the epitope recognized by antibodies in human and monkey serum, alpha-linked galactose was removed from the cells surface by treatment with alpha-galactosidase. Specifically, primary porcine endothelial cells were harvested from culture by trypsinization (trypsin obtained from BioWitaker, Walkersville, MD), neutralized with culture media, washed once with phosphate buffered saline (PBS) and once with 20 mM sodium acetate in  
10 PBS (pH 5.8). Coffee bean alpha-galactosidase (Sigma, St. Louis, MO) was added to cells (500 milliunits of enzyme/ $1 \times 10^6$  cells) and incubation was carried out for 2 hours at 37°C in 200 mM sodium acetate in PBS (pH 5.8). After enzyme incubation, the cells were washed twice with PBS and resuspended at  $2 \times 10^6$  cells/ml in culture medium. The cells were analyzed for viability by assay with MTT as described in Example 3. The results of this  
15 assay are illustrated in Figure 2. The data demonstrate that the enzyme treatment did not have an effect on the viability of the porcine endothelial cells.

Incubation of enzyme-treated cells with fluorescein labeled *Griffonia simplicifolia* I (GS-I FITC), a lectin which binds specifically to terminal alpha-galactosyl epitopes, showed that the enzyme was effective in removing the alpha-galactosyl groups on the cell surface  
20 using 0.5 units/ml with  $1 \times 10^6$  cells at 37°C for 2 hours. Binding of FITC labeled GS-I9 was assessed directly (GS-I) by flow cytometry, whereas binding of anti-SLA was assessed with a FITC labeled anti-mouse IgG (ANTI-SLA), and interaction of natural antibodies was determined by employing species-specific FITC-labeled anti-IgG or anti-IgM secondary antibodies (HUMAN IgG, HUMAN IgM, MONKEY IgG and MONKEY IgM). A control  
25 incubation with secondary antibody alone was performed to determine background. Only the untreated cells bound GS-I FITC as revealed by flow cytometry (See Figure 3A, GS-I). Unaltered binding of an anti-SLA monoclonal antibody to MHC class I molecules (See Figure 3B, ANTI-SLA) after enzyme treatment indicated that protease contamination of the enzyme did not account for the decreased GS-I binding. The removal of the alpha-  
30 galactosyl epitope by enzymatic digestion diminished the binding of the natural antibodies present in human and monkey sera. Both IgG and IgM binding was decreased in human serum (See Figures 3C and 3D, HUMAN IgG and HUMAN IgM) and in monkey serum (See Figures 3E and 3F, MONKEY IgG and MONKEY IgM) when detected with species specific anti-IgG FITC or anti-IgM FITC labeled secondary antibodies.

35 The extent of removal by alpha-galactosidase of epitopes recognized by natural antibodies is shown in Table I. Removal of alpha-linked galactose from endothelial cells by alpha-galactosidase decreased their reactivity with human and monkey natural antibodies by 59% to 90%. The reactivity of both IgG and IgM in the human and monkey sera were markedly affected by the removal of this single epitope.

TABLE I

Median fluorescence intensity of treated and untreated porcine endothelial cells incubated with human or monkey serum and detected with anti-IgG-FITC or anti-IgM-FITC secondary antibody.

5

Treatment	Serum	Secondary Antibody	Fluorescence Intensity
None	human	anti-IgG	126.55
Alpha-galase	human	anti-IgG	51.90 (59%) <sup>1</sup>
None	human	anti-IgM	69.91
Alpha-galase	human	anti-IgM	20.54 (71%)
None	monkey	anti-IgG	846.92
Alpha-galase	monkey	anti-IgG	83.40 (90%)
None	monkey	anti-IgG	57.01
Alpha-galase	monkey	anti-IgM	11.47 (80%)

<sup>1</sup> Numbers in parentheses represent the decrease in median fluorescence intensity observed after treatment with alpha-galactosidase.

10 Following enzyme treatment, the efficacy of removal of the alpha-galactosyl epitopes was also assessed. The cells were stained with fluorescein-labelled *Griffonia simplicifolia* I (GS-I). For staining, cells were incubated with labelled GS-1 (commercially obtained from EY Labs) for 30 minutes on ice. The stained cells were subjected to FACS analysis to determine the density of the reactive epitope on the cell surface at increasing times after digestion.

15 The results of the experiment are shown in Figure 4. The data indicates that alpha-galactosidase treatment of porcine endothelial cells can remove greater than 95% of the cell surface alpha-galactosyl epitopes. This greatly reduced level of expression of the epitope on the cell surface persists for several hours following enzyme treatment. Even by 48 hours after treatment, the level of surface expression of the epitope is still diminished by about 20 60% compared to untreated controls. This example demonstrates that alpha-galactosidase treatment of porcine endothelial cells is effective at removing cell surface alpha-galactosyl epitopes, and that the epitopes are not reexpressed for several hours to days following enzyme treatment.

25

**EXAMPLE 2:            Removal of Galactosyl( $\alpha$ 1,3)Galactose Epitopes Inhibits  
Binding of Natural Antibodies to Endothelial Cells**

In this example, the effect of removing alpha-galactosyl epitopes from the surface of  
5 porcine endothelial cells on subsequent binding of human and animal sera to the cells was  
examined. Porcine endothelial cells were isolated and treated with alpha-galactosidase to  
remove cell surface alpha-galactosyl residues, as described in Example 1. Immediately  
following treatment, the treated cells and untreated control cells were incubated with serum  
from cynomolgus monkey, human, mouse and pig. Sera were typically used at a 1:10  
10 dilution. Binding of antibodies to the cells was assessed using species-specific fluorescein-  
labelled anti-IgG or anti-IgM secondary antibodies (to assess the binding of IgG or IgM  
antibodies, respectively, within the sera). A control incubation with secondary antibody  
alone was performed to determine background labelling. Staining of the cells with the  
labelled secondary antibody was assessed by FACS analysis.

15            The results of the experiment are illustrated in Figure 5. Neither mouse nor porcine  
sera exhibited readily detectable binding to either untreated or enzyme treated porcine  
endothelial cells. In contrast, both human and monkey sera exhibited strong binding to  
untreated porcine endothelial cells. The predominant isotype detected was IgG, although low  
levels of IgM binding were also observed. These results confirm the presence of natural  
20 antibodies specific for porcine cells in human and monkey sera. Significantly, treatment of  
the porcine cells with alpha-galactosidase prior to incubation with the sera greatly reduced the  
subsequent binding of natural antibodies to the porcine cells. This effect was seen for both  
IgG and IgM isotypes. These results demonstrate that antibodies directed against alpha-  
galactosyl epitopes represent a major component of anti-porcine natural antibodies in human  
25 and nonhuman primate sera and, moreover, indicate that treatment of porcine cells with  
alpha-galactosidase can inhibit their recognition by these natural antibodies.

**EXAMPLE 3:            Removal of Galactosyl( $\alpha$ 1,3)Galactose Epitopes Inhibits  
Natural Antibody-Mediated Cytotoxicity**

30            Sera from humans was found to be cytotoxic to porcine endothelial cells in the  
presence of exogenously added rabbit complement as detected with a colorimetric assay for  
cell viability employing MTT as described in this Example. Porcine endothelial cells were  
incubated with alpha-galactosidase for 2 hours at 37°C before treatment with heat-inactivated  
serum with or without 10% rabbit complement. Cell viability was measured by the MTT  
35 assay as described below. The results of the experiment are illustrated in Figure 6. The  
absorbance obtained for cells treated with bovine serum and complement (CONTROL) is  
taken as 100%. The absorbance obtained for cells killed with alcohol (DEAD) is also given.  
Complement alone or in the presence of bovine serum had no effect on cell viability. The



cytotoxic effect of serum is dependent upon concentration. Thus, natural antibodies bind to porcine cells and are capable of killing the cells in the presence of complement.

Variations among seven individuals (humans) were apparent in the amount of natural antibodies in the sera. Porcine endothelial cells were incubated with 10% human serum from seven individuals followed by detection by flow cytometric analysis with human specific IgG or IgM FITC labeled secondary antibody. By flow cytometric analysis, seven different individuals displayed various degrees of IgG and IgM reactivity (mean fluorescence intensity, Figure 7). Variations in the ability of various human sera to kill porcine endothelial cells were also observed, but did not clearly correlate to the levels of natural antibodies in these individuals.

In this example, the effect of removing alpha-galactosyl epitopes from the surface of porcine endothelial cells on the ability of human sera, together with complement, to mediate cytotoxicity was determined. For the cytotoxicity assay, porcine endothelial cells, either untreated or treated with alpha-galactosidase as described in Example 1, were aliquoted into 1.5 ml microcentrifuge tubes ( $1 \times 10^6$  cells in 0.5 ml). Serum (either human or, as a control, bovine) was added to the tubes at an appropriate concentration (e.g., 10-20%), followed by addition of rabbit complement (obtained from Pel-Freeze, Rogers, NY) at a dilution of 1:5. DMEM (high glucose) with heat-inactivated fetal calf serum was added to bring the final volume to 1 ml. The tubes were incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. At the end of the incubation period, the tubes were centrifuged at 800 g for 5 minutes. The medium was aspirated off gently to avoid disruption of the pellet. Cells were then washed with 1 ml of Hanks buffered salt solution (Gibco, Grand Island, NY). The pellet was then resuspended in 0.5 ml of a 2.5 mg/ml stock solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) and an additional 0.5 ml of Hanks buffered salt solution was then added to each tube. MTT was prepared fresh on the day of the assay in DMEM (high glucose) and 10% fetal calf serum. Cells were incubated with MTT for 4 hours at 37°C in 5% CO<sub>2</sub>. After incubation, cells were centrifuged and washed twice with PBS. The MTT crystals were solubilized in 0.5 ml of acidified isopropanol (pH 3.0) and 100 µl from each tube was transferred to a 96-well microtiter plate. Each well was measured using an automatic plate reader with a 562 nm test wavelength.

In a first experiment using untreated porcine endothelial cells, the cells were incubated with either no serum, heat inactivated bovine serum or heat inactivated human serum, in the presence or absence of rabbit complement as described above. Cell viability was measured using the triazolium salt, MTT, as described above. As shown in Figure 8, neither complement alone nor bovine serum in the presence or absence of complement were cytotoxic. In contrast, human serum in the presence (but not the absence) of complement was capable of greatly reducing cell viability, indicating that natural antibodies within the serum are cytotoxic to the untreated porcine cells in a complement dependent manner.

In the next experiment, porcine endothelial cells were treated with alpha-galactosidase, as described in Example 1, to remove cell surface alpha-galactosyl residues. Following treatment, the treated cells or untreated control cells were incubated with human serum (10% or 20%) in the presence of rabbit complement for 4 hours as described above.

5 Control cells were incubated with bovine serum and complement. Cell viability was measured by the MTT assay, as described above. The absorbancy reading obtained for untreated cells incubated with bovine serum was taken as 100%, and viability of cells incubated with human serum was measured relative to this. As illustrated in Figure 9,

10 incubation of untreated porcine cells with human sera and complement reduced their viability by approximately 70% relative to control cells. However, treatment of the cells with alpha-galactosidase prior to incubation with human sera and complement restored full cell viability, indicating that removal of alpha-galactosyl residues from the surface of the porcine cells substantially eliminates the cytotoxicity of human natural antibodies, in combination with complement, against porcine cells.

15

**EXAMPLE 4:**      Inhibition of Alpha-1,3-Galactosyltransferase Activity in a Cell  
for Transplantation Using Antisense Nucleic Acid

Expression of galactosyl( $\alpha$ 1,3)galactose epitopes on the surface of a cell can be

20 reduced or substantially eliminated by inhibiting the activity of an alpha-1,3-galactosyltransferase enzyme in the cell. One method for inhibiting the activity of the enzyme utilizes a nucleic acid which is antisense to a region of the mRNA encoding the enzyme (i.e., antisense to a coding region of the gene for the enzyme). An oligonucleotide having a sequence which is antisense to mRNA encoding a UDP galactose-alpha-1,3-

25 galactosyltransferase, is designed based upon the rules of Watson-Crick base pairing and synthesized by standard techniques, e.g. using an automated DNA synthesizer. For example, three 20-mer oligonucleotides having sequences which are antisense to the following nucleotide positions of an alpha-galactosidase mRNA can be synthesized (nucleotide positions are relative to the start site of translation at position 0): nucleotide positions -15 to

30 +5 (surrounding the translation start site), +6 to +25 and +101 to +120. Suitable antisense oligonucleotide sequences, designed based upon the sequence of either the murine alpha-galactosyltransferase cDNA (disclosed in Larsen, R.D. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:8227-8231) or the bovine alpha-galactosyltransferase cDNA (disclosed in Joziassse, D.H. et al. (1989) *J. Biol. Chem.* 264:14290-14297), are as follows (in 5' to 3' orientation):

35

Mouse

(1 (-15 to +5): ATCATGAAAATCTTAGGTCC

(SEQ ID NO: 1)

2 (+6 to +25): GGAGATCTTGAAGCATAGTG

(SEQ ID NO: 2)

3 (+101 to +120): TCCCTTGACATTTCATTATTT (SEQ ID NO: 3)

#### Cow

- 1 (-15 to +5): TTCATTATTTTCTCCTCATC (SEQ ID NO: 4)  
 5 2 (+6 to +25): GAATCACTTTTCCTTTGACA (SEQ ID NO: 5)  
 3 (+101 to +120): GTTCTTGATGGGTTTATCC (SEQ ID NO: 6)

To inhibit the activity of the enzyme in porcine hepatocytes, the three oligonucleotides, each at a concentration of 5  $\mu$ M, are added to porcine hepatocyte cultures. After 2 days, the expression of cell surface alpha-galactosyl epitopes is evaluated by FACS analysis using FITC-labelled GS-I lectin as described in Example 3. The cells ( $1 \times 10^8$ ) are transplanted into a monkey by infusion into an indwelling catheter in the portal vein (in a suspension of 100 ml over 30 minutes). At days 1 through 5 after surgery, a solution of the antisense nucleotides (1 ml of a 5  $\mu$ M solution of each nucleotide) is infused through the catheter. The success of the transplant can be assessed by determining the level of porcine albumin in the monkey serum at intervals after transplantation by conventional techniques. At termination of the experiment, the porcine hepatocytes can be localized by immunohistochemistry using pig specific primary antibodies and biotinylated secondary antibody followed by detection with streptavidin peroxidase.

#### Other Embodiments

While the invention has been described in particular with regard to xenogeneic transplantation, the invention can be applied to other clinical situations involving natural antibody-mediated hyperacute rejection. For example, natural antibodies play a role in the rejection of certain allografts, such as allografts transplanted across an ABO blood group mismatch. Similar to the epitopes on nonprimate cells recognized by natural antibodies in humans and other nonhuman primates, the A and B blood group antigens are composed of carbohydrate epitopes. Accordingly, the methods of the invention can similarly be applied to altering, reducing or substantially eliminating the expression of A and/or B blood group antigens on an allogeneic cell to be transplanted into an ABO incompatible recipient.

Additionally, in humans the exposure of cryptic alpha-galactosyl epitopes on the surface of certain cells is thought to be involved in autoimmune responses. While terminal alpha-galactosyl epitopes are not normally expressed on human cells, internal alpha-galactosyl epitopes can become exposed on certain cell types, such as erythroid cells or thyroid cells, either as a result of aging or disease (e.g., exposure on erythrocytes as a result of a hematological disorder). Inappropriate exposure of these cryptic epitopes leads to destruction of the the cells (e.g., erythrocytes), presumably mediated by natural antibodies in the individual directed against the epitope. Moreover, this mechanism has been directly implicated in the premature destruction of erythrocytes in sickle cell anemia. (Galili, U. et al.

(1987) *J. Biol. Chem.* 262:4683-4688; Galili, U. et al. (1986) *J. Clin. Invest.* 77:27-33).

Accordingly, the methods of the invention for altering, reducing or eliminating the expression of alpha-galactosyl epitopes on the surface of cells can also be applied therapeutically in sickle cell anemia and other disorders associated with inappropriate expression of alpha-

5 galactosyl residues on cells to inhibit the binding of natural antibodies to this cryptic epitope. For example, for treatment of a hematological disorder, erythrocytes can be removed from a subject, treated *in vitro* with an alpha-galactosidase and returned to the subject.

Alternatively, a nucleic acid (e.g., recombinant expression vector) which is antisense to an alpha-galactosyltransferase gene can be introduced into a hematopoietic stem cell of the  
10 subject to inhibit the expression of the epitope on hematologic cells.

### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention  
15 described herein. Such equivalents are intended to be encompassed by the following claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Albert Edge

10

(ii) TITLE OF INVENTION: Modified Cells and Methods for Inhibiting  
Hyperacute Rejection of Xenogeneic  
Transplants

(iii) NUMBER OF SEQUENCES: 6

15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: LAHIVE &amp; COCKFIELD

(B) STREET: 60 State Street, suite 510

(C) CITY: Boston

(D) STATE: Massachusetts

(E) COUNTRY: USA

20

(F) ZIP: 02109-1875

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

25

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII text

(vi) CURRENT APPLICATION DATA:

30

(A) APPLICATION NUMBER:

(B) FILING DATE: 17-MAY-1995

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

35

(A) APPLICATION NUMBER: US 08/253,782

(B) FILING DATE: 03-JUN-1994

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

40

(A) NAME: Jean M. Silveri

(B) REGISTRATION NUMBER: P-39,030

(C) REFERENCE/DOCKET NUMBER: DNI-007PC

(ix) TELECOMMUNICATION INFORMATION:

45

(A) TELEPHONE: (617)227-7400

(B) TELEFAX: (617)227-5941

## (2) INFORMATION FOR SEQ ID NO:1:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCATGAAAA TCTTAGGTCC

20

5 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAGATCTTG AAGCATAGTG

20

20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

25 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

35 TCCCTTGACA TTCATTATTT

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

50

TTCATTATTT TCTCCTCATC

20

(2) INFORMATION FOR SEQ ID NO:5:

55 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATCACTTT TCCTTTGACA

20

10 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTTCTTGAT GGGTTTATCC

20

25

CLAIMS

1. A cell which, in unmodified form, expresses at least one epitope on a cell surface antigen which is bound by natural antibodies when the cell is transplanted into a recipient, wherein the cell is modified to alter, reduce or substantially eliminate expression of the epitope on the cell surface.
2. The cell of claim 1, which is porcine.
3. The cell of claim 1, wherein the epitope is a carbohydrate.
4. A cell which, in unmodified form expresses at least one galactosyl( $\alpha$ 1-3)galactose epitope on a cell surface antigen, wherein the cell is modified to alter, reduce or substantially eliminate expression of the epitope on the cell surface.
5. The cell of claim 4, wherein the cell is modified to reduce or substantially eliminate expression of the epitope on the cell surface.
6. The cell of claim 5, which is a nonprimate mammalian cell.
7. The cell of claim 5, which is a prosimian or New World Monkey cell.
8. The cell of claim 6, which is a porcine cell.
9. The cell of claim 5, which is modified by introduction of a nucleic acid which is antisense to a regulatory or coding region of an  $\alpha$ 1-3-galactosyltransferase gene into the cell.
10. The cell of claim 4, which is modified by contacting the cell with at least one molecule which binds to the epitope prior to transplantation.
11. The cell of claim 5, which is selected from the group consisting of an endothelial cell, a hepatocyte, a pancreatic islet cell, a skeletal myocyte, a skeletal myoblast, a cardiac myocyte, a cardiac myoblast, a fibroblast, an epithelial cell, a neuronal cell, a bone marrow cell, a hematopoietic cell and a lymphoid cell.
12. The cell of claim 5, which is within a tissue or an organ.
13. The cell of claim 5, which is further modified to express a gene product.



14. The cell of claim 5, which, in unmodified form, further expresses at least one second cell surface antigen which stimulates a cellular immune response against the cell in a recipient, wherein the cell is further modified to alter, reduce or substantially eliminate expression of the second cell surface antigen.
15. The cell of claim 14, wherein the second cell surface antigen is an MHC class I antigen.
16. The cell of claim 15, which is further modified by contacting the cell prior to transplantation with an antibody, or fragment thereof, which binds to the MHC class I antigen.
17. A porcine cell which is modified to alter, reduce or substantially eliminate expression of at least one galactosyl( $\alpha$ 1-3)galactose epitope on a cell surface antigen.
18. The porcine cell of claim 17, which is further modified by contacting the cell prior to transplantation with an antibody, or fragment thereof, which binds to at least one porcine MHC class I antigen on a surface of the cell.
19. A method for reducing the immunogenicity of a cell for transplantation into a recipient, wherein the cell in unmodified form expresses at least one epitope on a cell surface antigen which is bound by natural antibodies in the recipient, comprising contacting the cell with a first agent which alters, reduces or substantially eliminates expression of the epitope on the cell surface such that when the cell is transplanted into a recipient, hyperacute rejection of the cell is inhibited.
20. The method of claim 19, wherein the epitope is a carbohydrate.
21. The method of claim 20, wherein the epitope is a galactosyl( $\alpha$ 1-3)galactose epitope.
22. The method of claim 21, wherein the first agent cleaves the galactosyl( $\alpha$ 1-3)galactose epitope from the cell surface.
23. The method of claim 22, wherein the first agent is an alpha-galactosidase.
24. The method of claim 21, wherein the first agent inhibits formation of the galactosyl( $\alpha$ 1-3)galactose epitope on the cell surface.

25. The method of claim 24, wherein the first agent inhibits the activity of an  $\alpha$ 1-3-galactosyltransferase within the cell.
- 5 26. The method of claim 25, wherein the first agent is a nucleic acid introduced into the cell which is antisense to a regulatory or coding region of an  $\alpha$ 1-3-galactosyltransferase gene.
- 10 27. The method of claim 26, wherein the nucleic acid is at least one oligonucleotide of 5-35 nucleotides in length.
28. The method of claim 26, wherein the nucleic acid is contained in a recombinant expression vector.
- 15 29. The method of claim 21, wherein the first agent binds to the galactosyl( $\alpha$ 1-3)galactose epitope and inhibits binding of natural antibodies to the epitope in a recipient.
30. The method of claim 29, wherein the first agent is a lectin.
- 20 31. The method of claim 29, wherein the first agent is an antibody, or fragment thereof, which binds to the epitope but does not activate complement or cause lysis of the cell.
32. The method of claim 21, further comprising, after contacting the cell with the first agent, administering the cell to a recipient.
- 25 33. The method of claim 32, further comprising administering to the recipient a second agent which inhibits T cell activity in the recipient.
- 30 34. The method of claim 21, wherein the cell in unmodified form expresses at least one second epitope on a second cell surface antigen which stimulates a cellular immune response against the cell in the recipient, the method further comprising contacting the cell with a second agent which alters, reduces or substantially eliminates expression of the second cell surface antigen.
- 35 35. The method of claim 34, wherein the second cell surface antigen is an MHC class I antigen.
36. The method of claim 35, wherein the second agent is at least one antibody, or fragment thereof, which binds to the MHC class I antigen.

37. The method of claim 34, further comprising, after contacting the cell with the first and second agent, administering the cell to a recipient.
- 5 38. The method of claim 37, further comprising administering to the recipient a third agent which inhibits T cell activity in the recipient.
39. An isolated nucleic acid comprising a nucleotide sequence which is antisense to a coding or regulatory region of an alpha-1,3-galactosyltransferase gene.
- 10 40. The nucleic acid of claim 39, which, when introduced into a porcine cell, inhibits the activity of a porcine alpha-1,3-galactosyltransferase in the cell.
41. The nucleic acid of claim 39, which is an oligonucleotide of 5-35 nucleotides in length.
- 15 42. The oligonucleotide of claim 41, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6.
- 20 43. The nucleic acid of claim 39, which is contained in a recombinant expression vector.

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FIG. 1A

HUMAN IgG

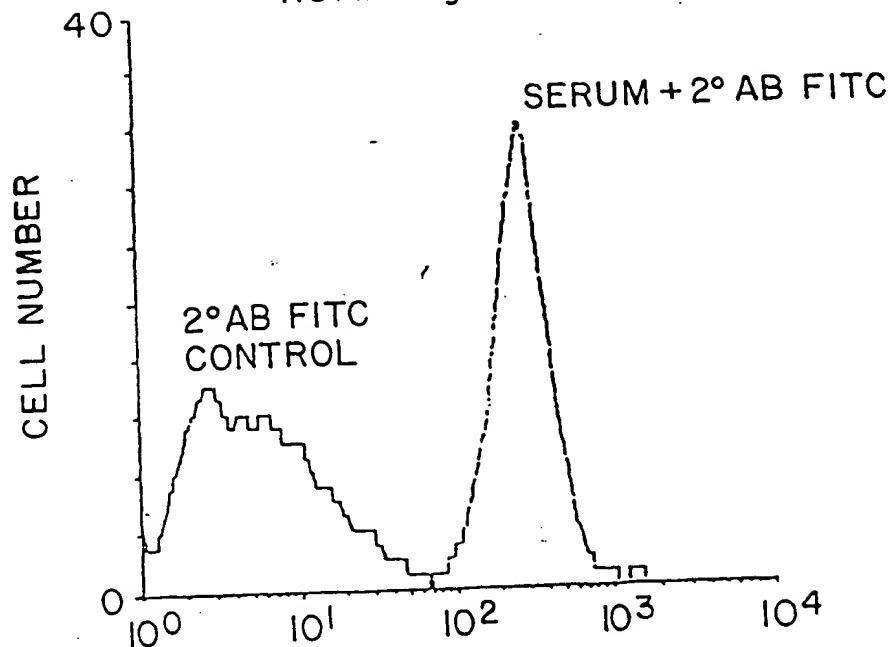
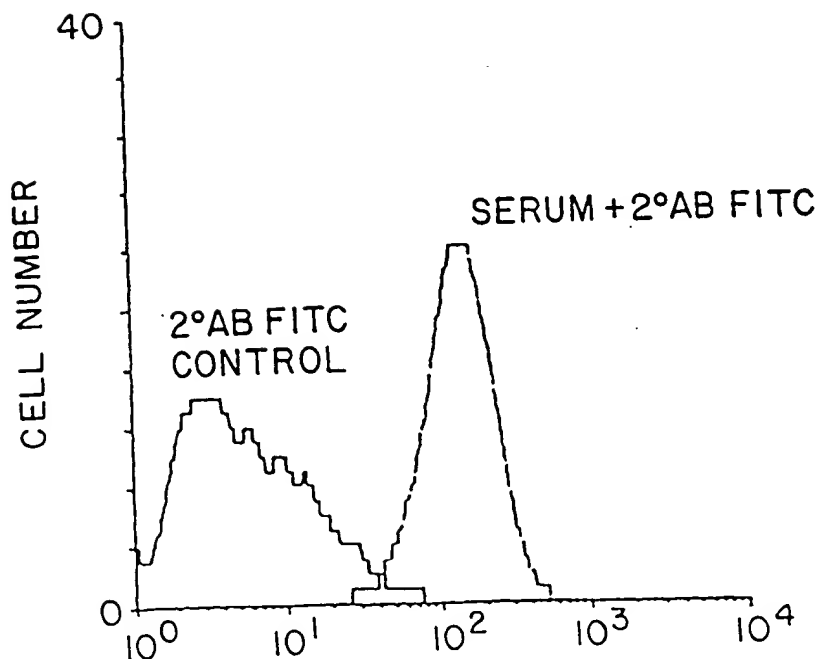
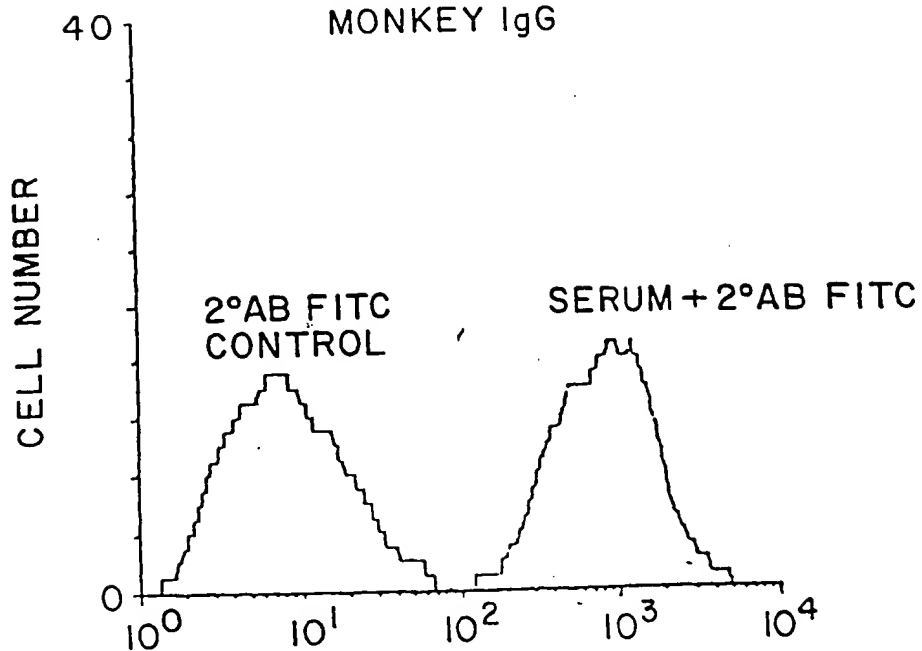


FIG. 1B

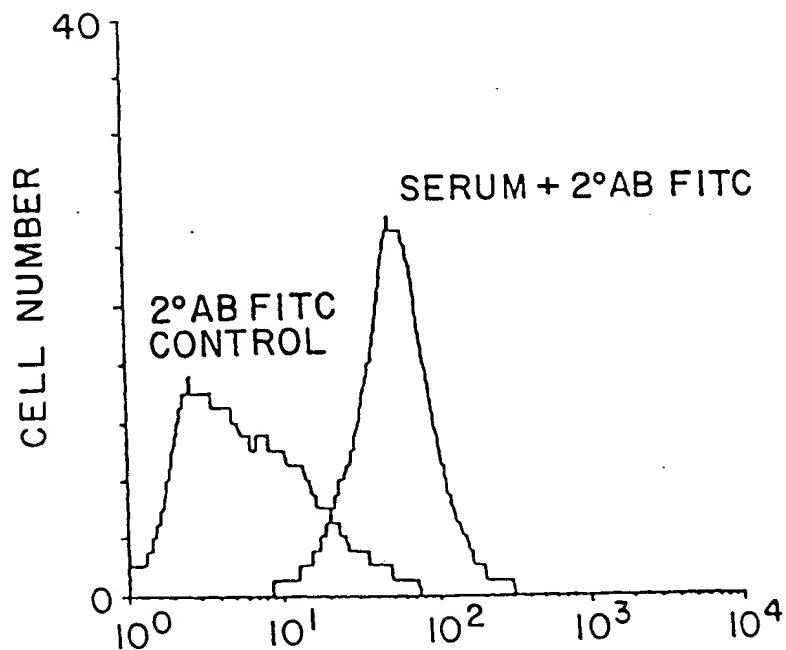
HUMAN IgM



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**FIG. 1C**  
MONKEY IgG**FIG. 1D**

MONKEY IgM



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FIG. 2

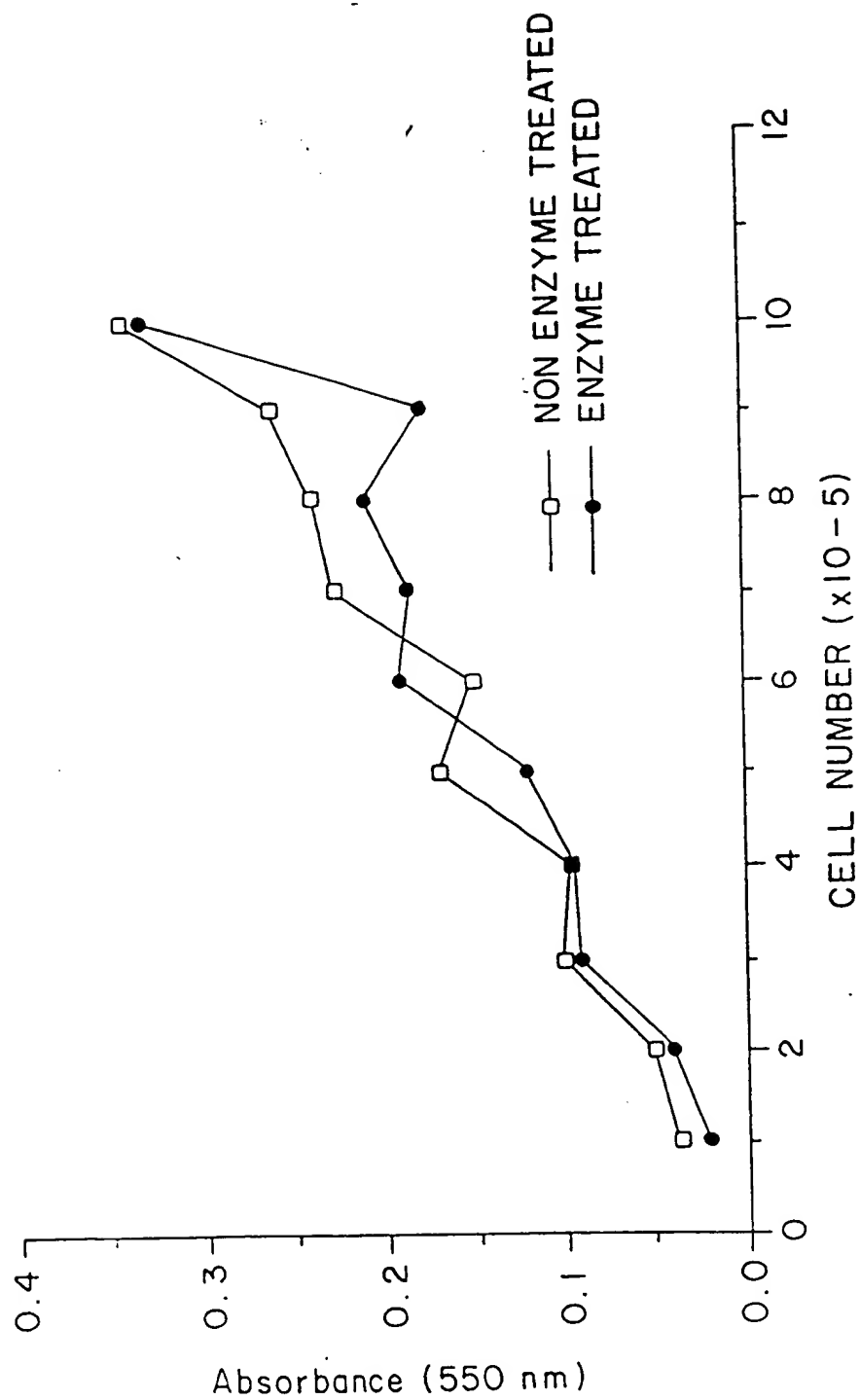


FIG. 3A

GS-I

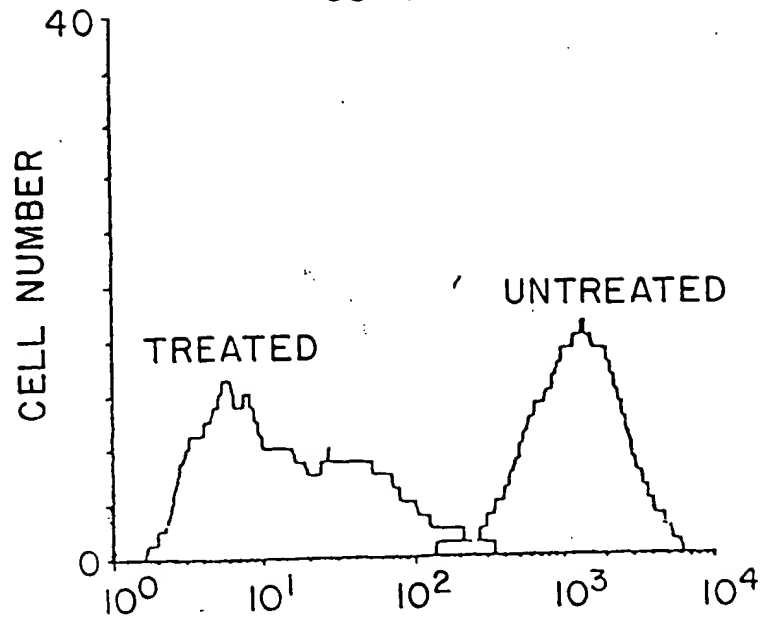


FIG. 3B

ANTI-SLA

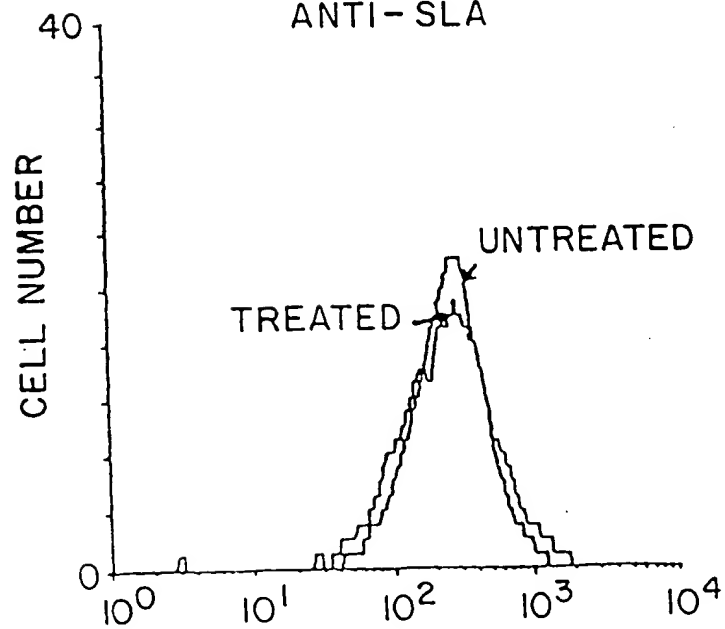


FIG.3C

HUMAN IgG

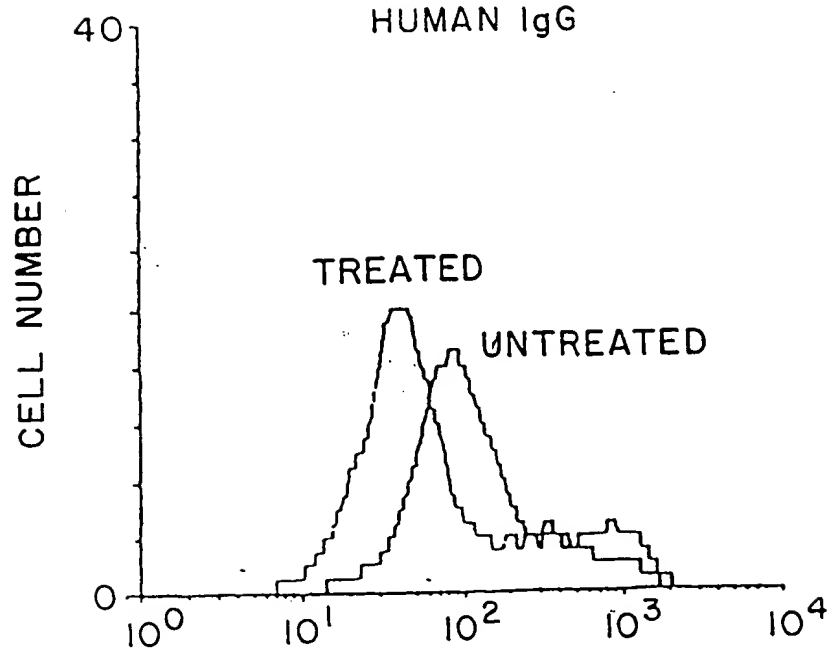
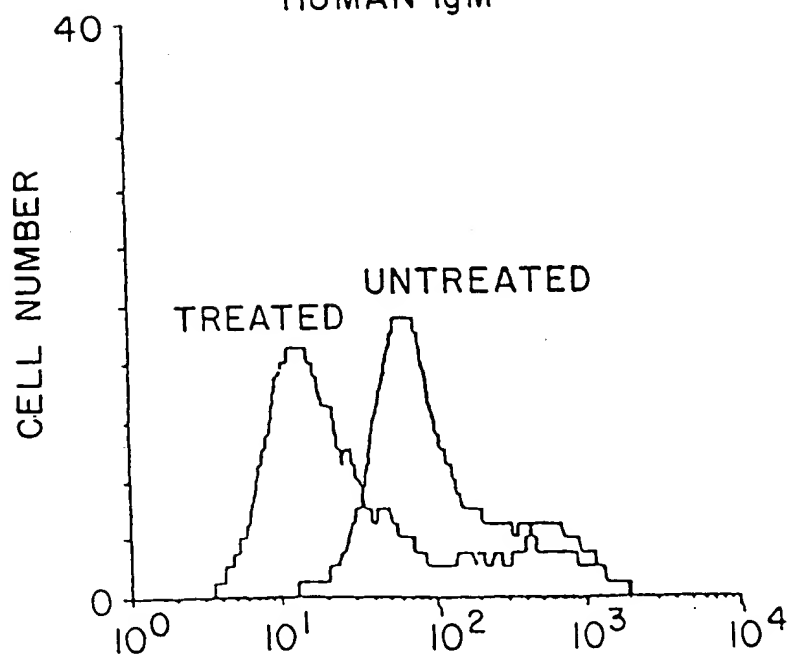


FIG.3D

HUMAN IgM





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FIG. 3E  
MONKEY IgG

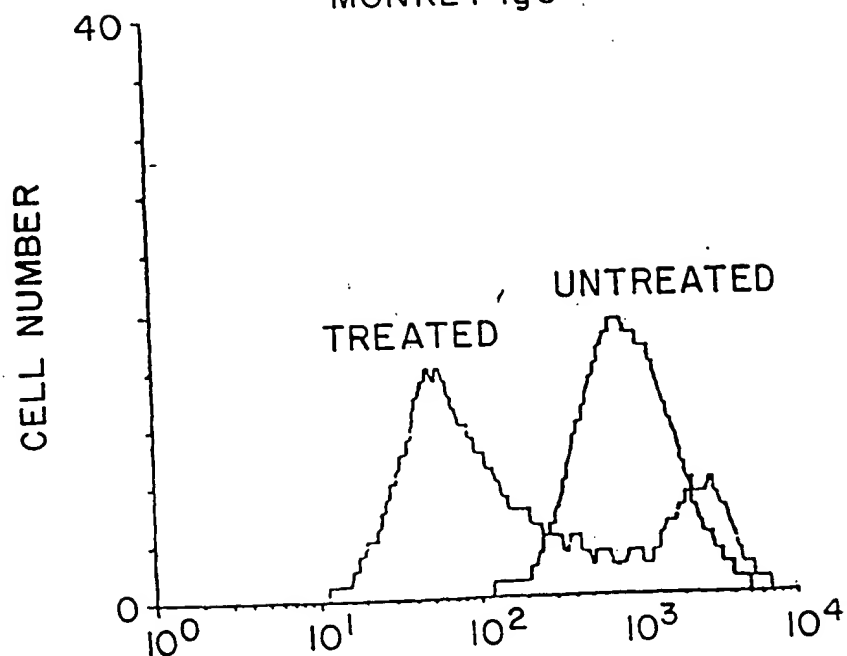
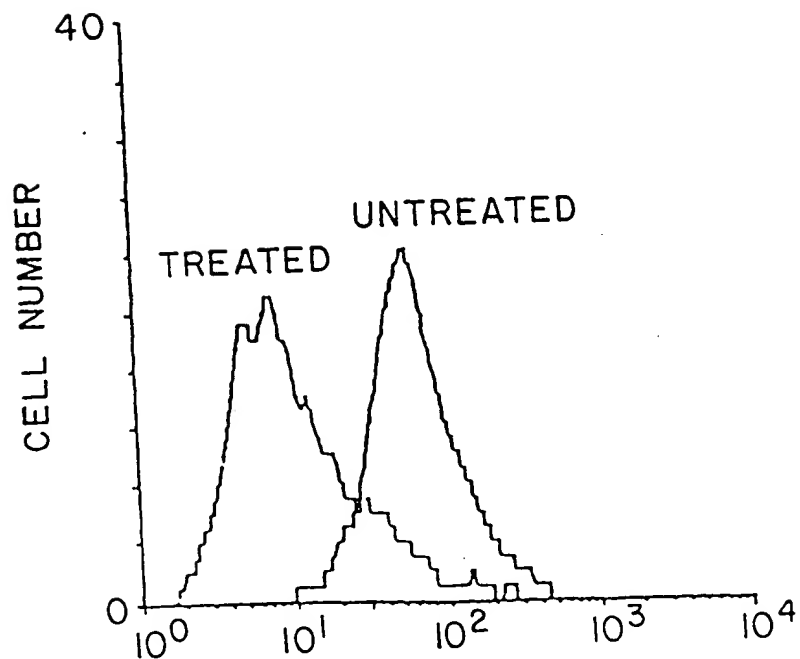
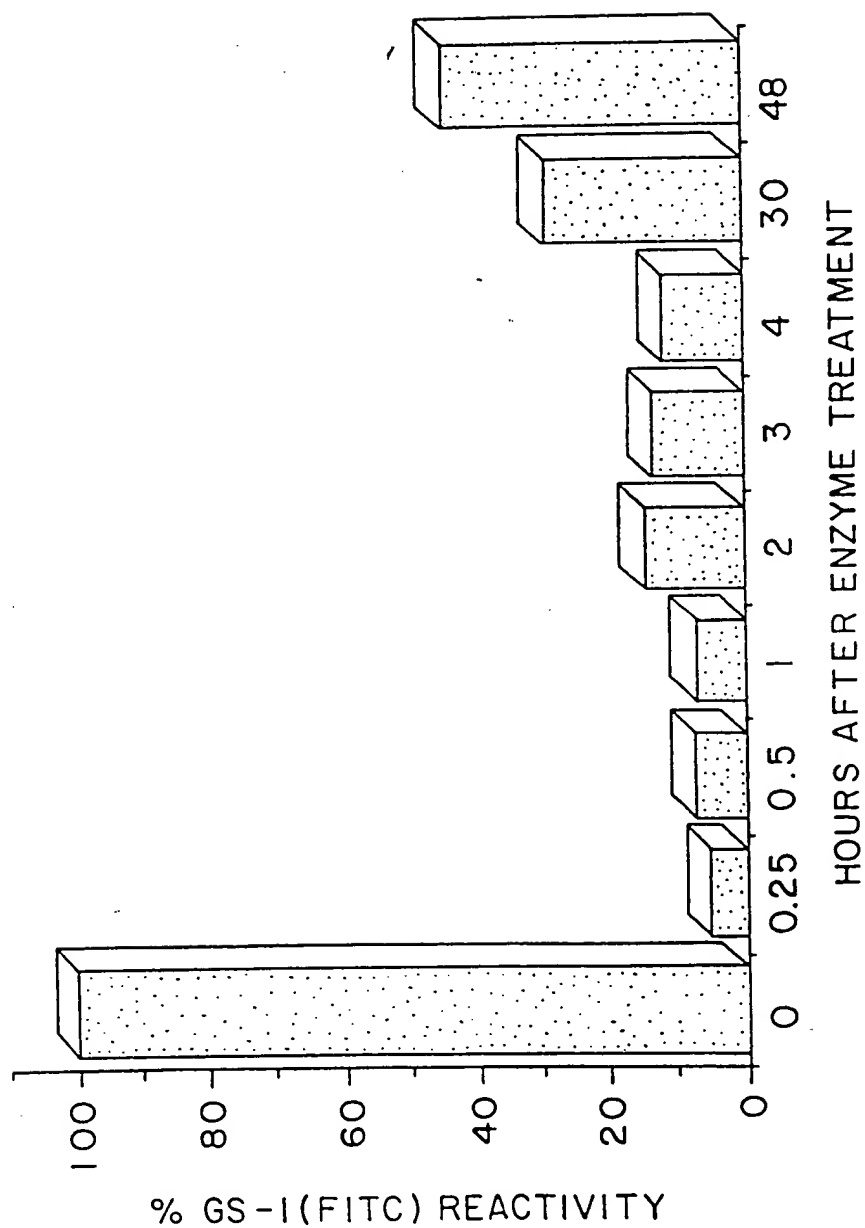


FIG. 3F  
MONKEY IgM



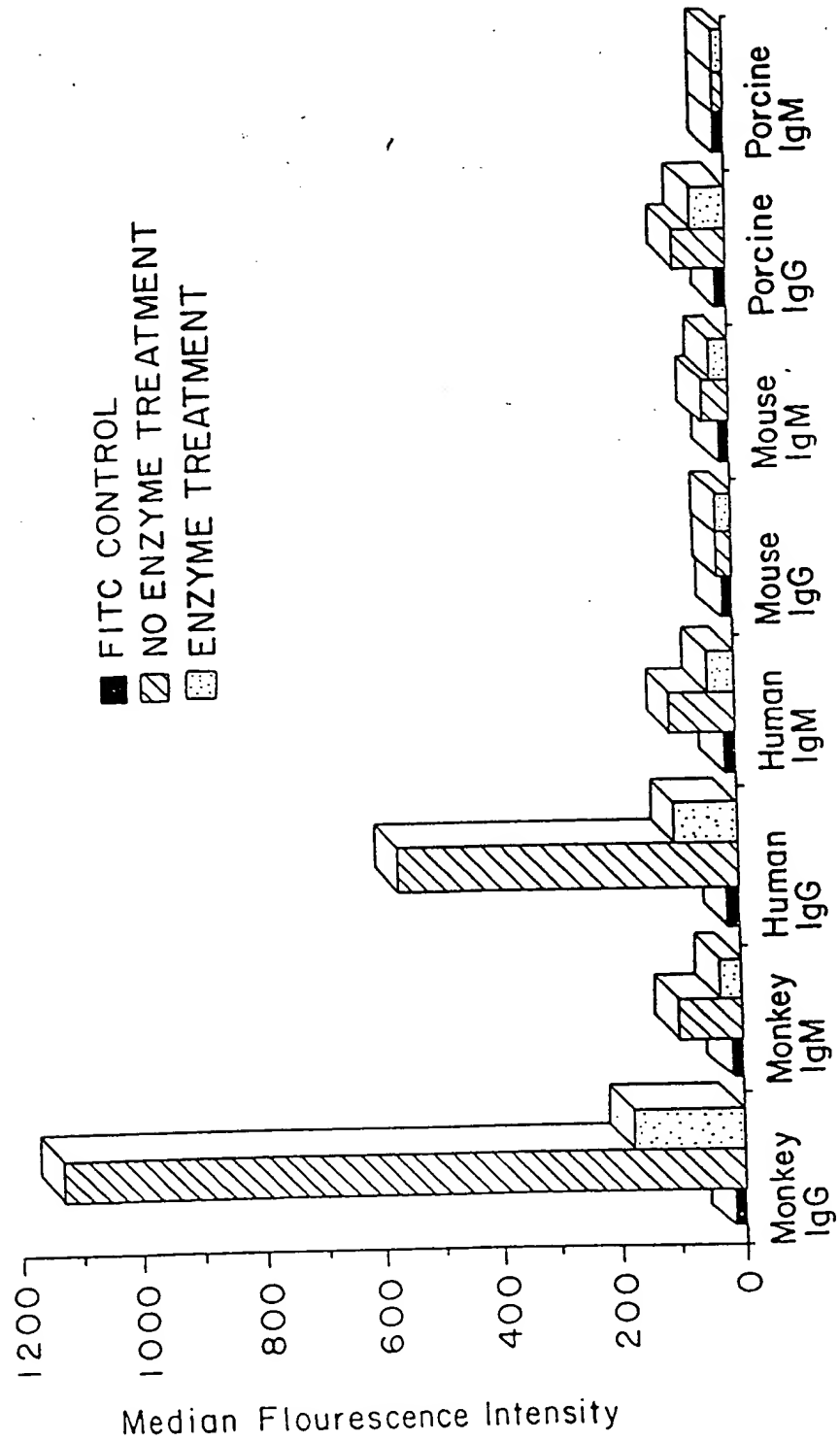
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FIG. 4



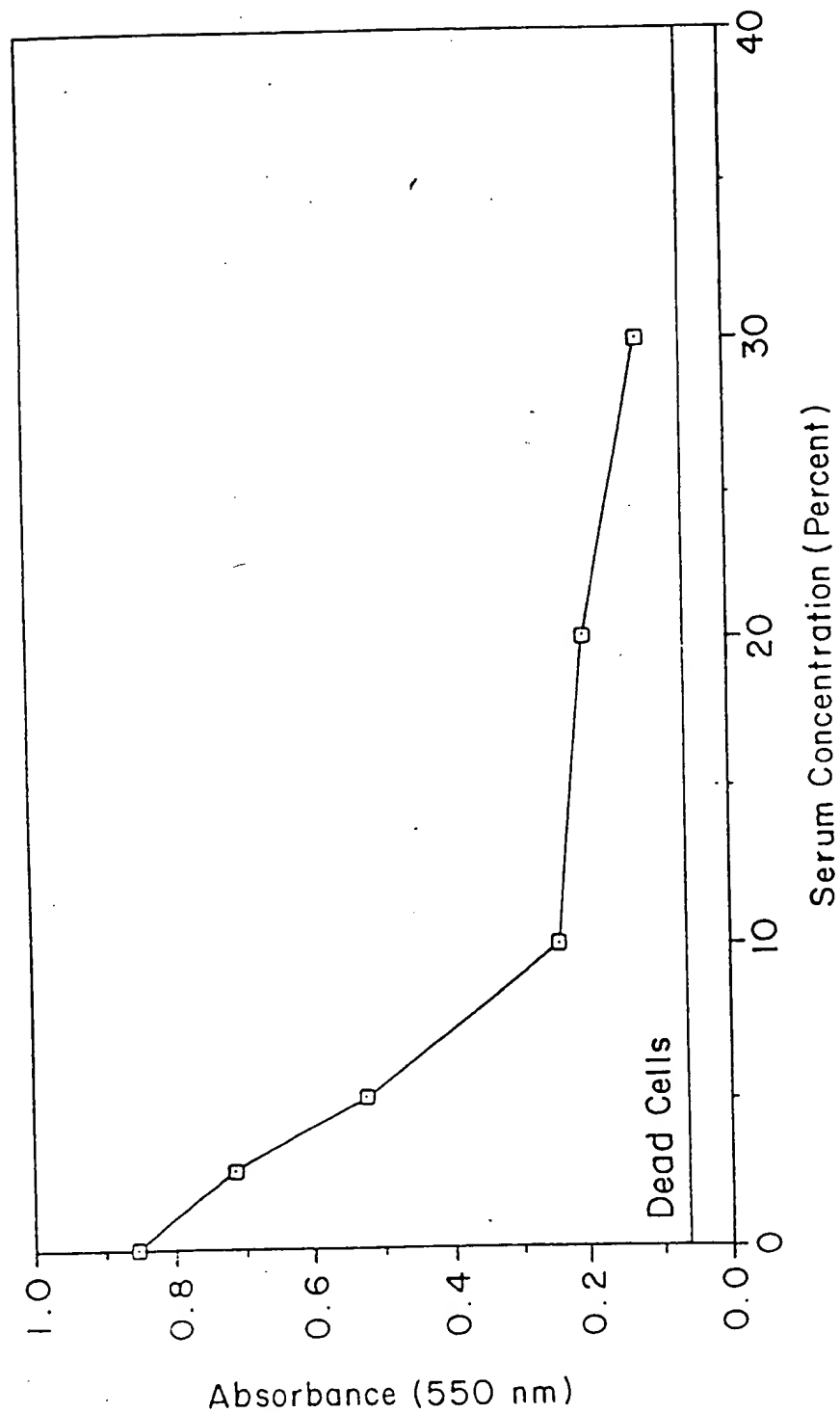
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FIG. 5



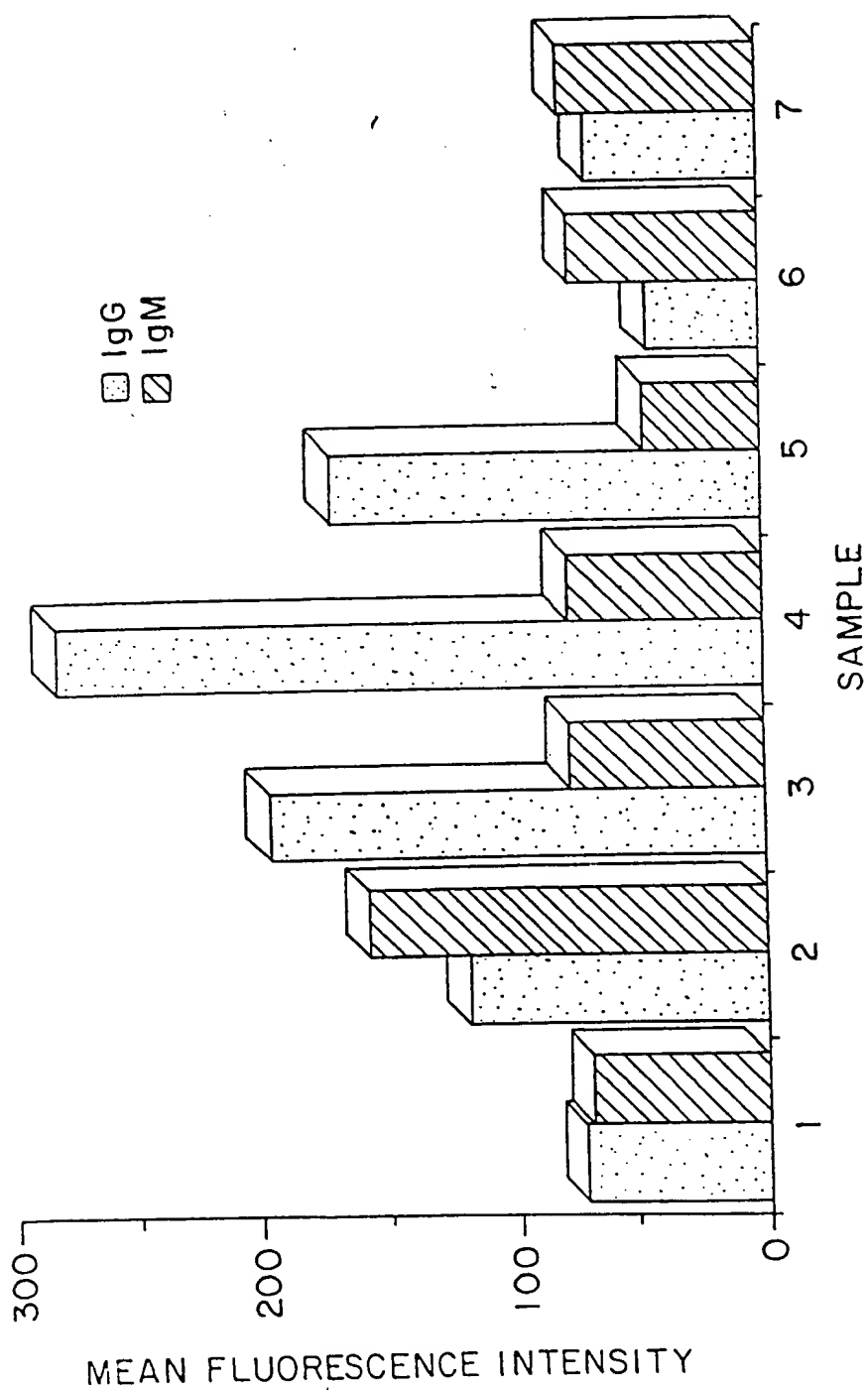
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FIG. 6



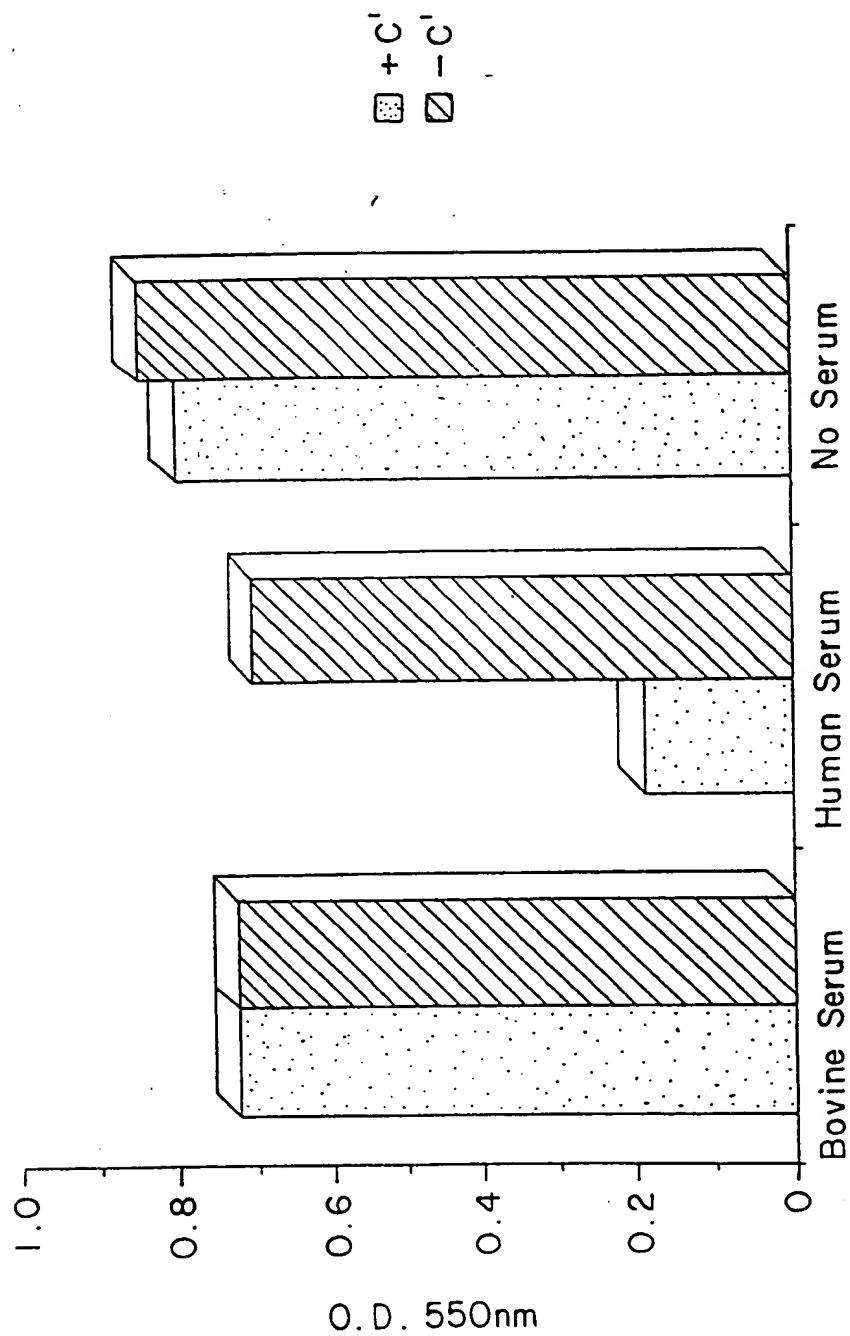
10/12

FIG. 7



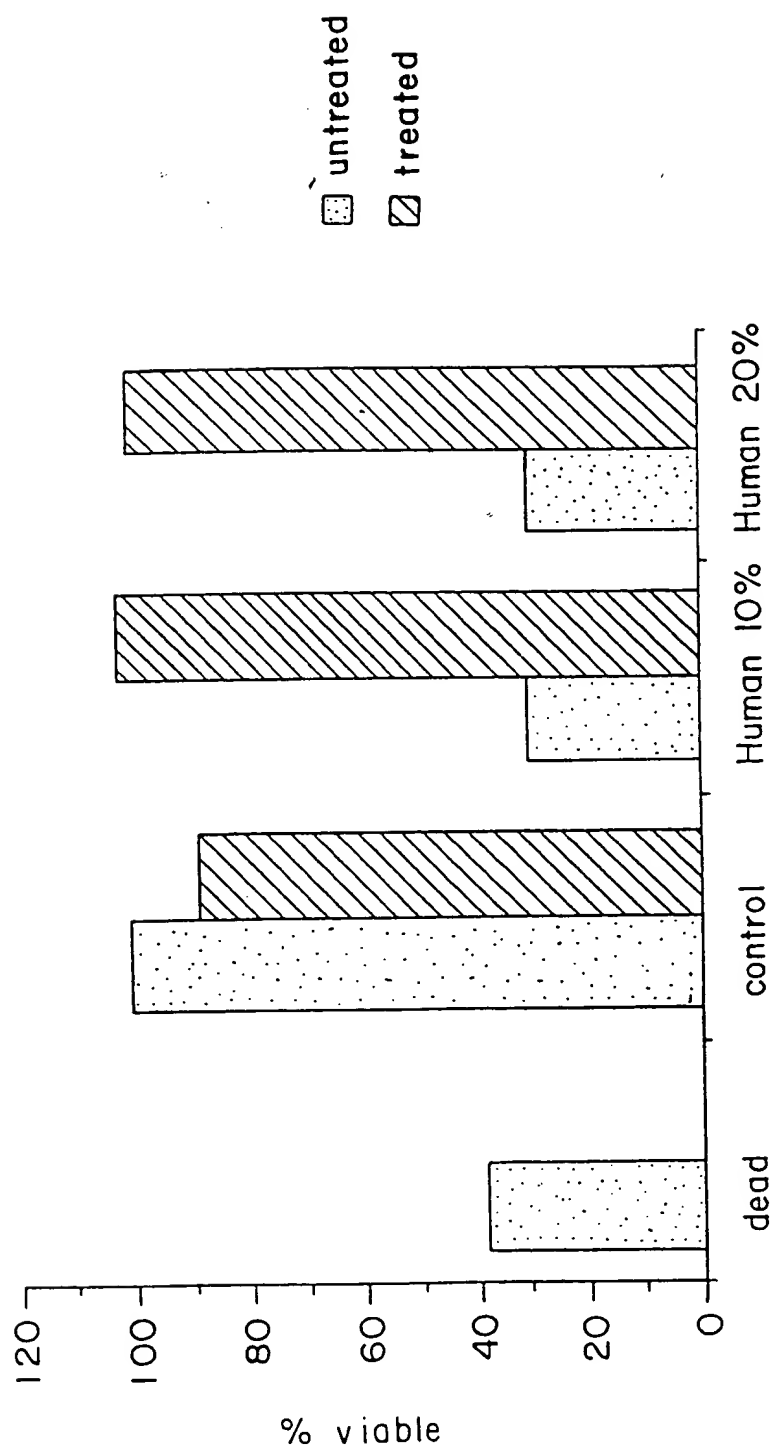
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FIG. 8



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FIG. 9



## INTERNATIONAL SEARCH REPORT

Int. .onal Application No

PCT/US 95/05973

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/11 C12N5/06  
//A61K38/47

C12N5/10

A61K35/407

A61K35/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, no. 23, 1 December 1993 WASHINGTON, DC, USA, pages 11391-11395, M. SANDRIN ET AL. 'Anti-pig IgM antibodies in human serum react predominantly with Gal(alpha1-3)Gal epitopes.' see abstract see page 11395, left column, line 46 - line 55	1-6,8,9, 11,12, 17, 19-21, 24-28, 32, 39-41,43
Y	---	14-16, 18, 34-37,42
	---	---

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*B\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*A\* document member of the same patent family

Date of the actual completion of the international search

21 September 1995

Date of mailing of the international search report

05.10.95

Name and mailing address of the ISA

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Authorized officer

Nooij, F



## INTERNATIONAL SEARCH REPORT

Int'l. Application No

PCT/US 95/05973

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL TRANSPLANTATION, vol. 3, no. 3, May 1994 NEW YORK, NY, USA, page 216 B. HERTEL-WULFF ET AL. 'Long-term survival of pancreatic islets in diabetic monkeys.' see abstract 20 ----	14-16, 18,34-37
Y	WO,A,94 02616 (UNIVERSITY OF MICHIGAN) 3 February 1994 see figure 2 ----	42
X	IMMUNOLOGY TODAY, vol. 14, no. 10, October 1993 AMSTERDAM, NL, pages 480-483, U. GALILI ET AL. 'Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans.' see page 482, left column, line 36 - line 50	1-9,11, 12,17, 19-21, 24-29, 31,32, 39-41,43
Y	----	14-16, 18, 34-37,42
Y	SCIENCE, vol. 252, no. 5013, 21 June 1991 WASHINGTON, DC, USA, pages 1700-1702, D. FAUSTMAN ET AL. 'Prevention of xenograft rejection by masking donor class I antigens.' see abstract see table 2 ----	14-16, 18, 34-37,42
P,X	TRANSPLANTATION, vol. 59, no. 1, 15 January 1995 BALTIMORE, MD, USA, pages 102-109, H. VAUGHAN ET AL. 'Biochemical studies of pig xenoantigens detected by naturally occurring human antibodies and the galactose-alpha(1-3)-galactose reactive lectin.' see abstract see page 108, left column, line 17 - line 32 ----	1-6, 8-12,17, 19-21, 24-32, 39,40,43
	----- -/--	

## INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 95/05973

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	IMMUNOLOGICAL REVIEWS, vol. 141, October 1994 COPENHAGEN, DENMARK, pages 169-190, M. SANDRIN ET AL. 'Gal.alpha(1,3)Gal, the major xenoantigen(s) recognised in pigs by human natural antibodies.' see page 185, line 25 - line 34 see table III -----	1-6, 8-12, 17, 19-30, 32, 39, 40, 43
P,X	WO,A,94 21799 (AUSTIN RESEARCH INSTITUTE) 29 September 1994  see page 8, line 7 - page 9, line 26 see examples 6,7 see claims -----	1-6, 8, 9, 17, 19-21, 24-28, 32, 39, 40, 43

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 95/05973

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9402616	03-02-94	US-A- 5324663	28-06-94
		AU-B- 4774793	14-02-94
		CA-A- 2140550	03-02-94
		EP-A- 0654082	24-05-95
WO-A-9421799	29-09-94	AU-B- 6279294	11-10-94

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/05973

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 32, 33, 37, 38  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 32, 33, 37 and 38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.